

# Effects of Carnosine and L-histidine on Viability and Expression of Pyruvate Dehydrogenase Kinase 4 in Human Glioblastoma Cells

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## Bibliographische Beschreibung

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### Referat:

Die Arbeit behandelt die Ergebnisse von Experimenten über die Wirkung des Dipeptides Carnosin ( $\beta$ -Alanyl-L-Histidin) und der Aminosäuren L-Histidin und  $\beta$ -Alanin auf Kulturen der humanen Zellreihen U87, T98G und LN405, welche von Zellen des malignen Hirntumors Glioblastoma multiforme abgeleitet sind. Die Vitalität der Zellen nach Inkubation mit Carnosin oder L-Histidin wurde anhand der Adenosintriphosphatproduktion und der Dehydrogenaseaktivität für Inkubationszeiträume von 24, 48 und 72 Stunden bestimmt. Dabei zeigte sich eine signifikant niedrigere Vitalität der mit Carnosin oder L-Histidin inkubierten Zellen gegenüber der unbehandelten Kontrolle. Dieser Effekt war bei L-Histidin stärker ausgeprägt. Bei Messungen der Laktatdehydrogenaseaktivität im Medium der Zellen, welche als Indikator für Zellnekrosen diente, zeigten nur die mit L-Histidin inkubierte Zellen Zeichen von Nekrose. Die gleichen Messungen wurden auch an humanen embryonalen Nierenzellen durchgeführt (HEK 293), wobei sich ein ähnliches Ergebnis feststellen ließ. In den drei Zellreihen wurde zudem mittels qRT-PCR die mRNA-Expression für die beiden Enzyme Carnosinase 1 und Carnosinase 2 bestimmt, welche L-Histidin von Carnosin abspalten. Im Vergleich mit Proben aus normalem Hirngewebe war die Expression beider Enzyme in den Glioblastomzellen deutlich geringer, wenngleich nachweisbar. Nachdem vorhergehende Studien [8] einen Anstieg der Expression von mRNA der Pyruvatdehydrogenasekinase 4 (PDK4) in mit Carnosin inkubierten Glioblastomzellen gezeigt hatten, wurde dieser Effekt hier auch mittels qRT-PCR in mit L-Histidin inkubierten Zellen nachgewiesen. Eine Wirkung von Carnosin oder L-Histidin auf ein Reporterogen des PDK4-Promoters wurde ebenfalls untersucht, wobei sich kein signifikanter Effekt nachweisen ließ.

## List of Abbreviations

Acetyl-CoA	Acetyl Coenzyme A
AMPK	AMP-activated protein kinase
ANSI	American National Standards Institute
AP2	Activating protein 2
ATP	Adenosine triphosphate
ATPGDP1	ATP-grasp domain containing protein-1
C/EBP $\beta$	CCAAT/enhancer-binding protein $\beta$
CBP	CREB-binding protein
CD	Cluster of differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2a
cDNA	Complementary DNA
CN	Carnosinase
CNS	Central nervous system
CTB	CellTiter-Blue Cell Viability Assay
CTG	CellTiter-Glo One Solution Assay
CTX	CytoTox-ONE homogenous membrane integrity assay
DNA	Desoxy ribonucleic acid
EC50	Half-maximal effective concentration
EGFR	Endothelial growth factor receptor
ERR	Estrogen related receptor
FBS	Fetal Bovine Serum
FOX	Forkhead-box-protein
GABRA1	Gamma-aminobutyric acid receptor alpha 1
GRE	Glucocorticoid responsive element
HIF	Hypoxia inducible factor
HPLC	High performance liquid chromatography
IDH1	Isocitrate dehydrogenase 1
IL-8	Interleukin 8
IRE	Insulin responsive element
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LDH	Lactate dehydrogenase
LOH	Loss of Heterozygosity
MGMT	O6-methylguanine-DNA methyltransferase

miRNA	Micro Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
NAD	Nicotinamide adenine dinucleotide
NEFL	Neurofilament light polypeptide
NFκB	Nuclear factor κB
Olig2	Oligodendrocyte lineage transcription factor 2
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PDGFRA	Alpha-type platelet-derived growth factor receptor
PDK	Pyruvate dehydrogenase kinase
PEPT	Oligopeptide transporter
PGC-1α	Peroxisome proliferator-activated receptor γ co-activator 1α
PHT1	Peptide/histidine transporter
PPARα	Peroxisome proliferator-activated receptor α
PTEN	Phosphatase and Tensin homolog
qRT-PCR	quantitative real time polymerase chain reaction
RARE	Retinoic acid response element
RNA	Ribonucleic acid
SLC	Solute carrier
SP1	Specificity protein 1
STAT5	Signal transducer and activator of transcription 5
SYT1	Synaptotagmin 1
TBP	TATA-box binding protein
TNF	Tumour necrosis factor
TP53	Tumour protein p53
tsp	Transcriptional start point
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WHO	World health organization
YKL	Chitinase-3-like protein

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# 1 Introduction

## 1.1 Overview

The dipeptide carnosine and the highly malignant central nervous system (CNS) tumour glioblastoma have both been subject of intensive and widespread research in recent years. Since treatment options for glioblastoma are still limited, evidence of tumour growth inhibition by carnosine opened a promising new pathway. First reports of an antineoplastic effect of carnosine in mice were published by Nagai and Suda in 1986 [1]. A similar result was obtained by Renner *et al.* who also demonstrated an inhibitory effect of carnosine on the growth of cell cultures of human glioblastoma [2, 3]. Analogous findings in other tumour cell cultures were presented by other researchers [4–6]. The works of Renner and Asperger suggested a crucial role of lowered ATP production and glycolysis in this effect [7], thus pointing out the regulatory enzymes of the citric acid cycle and glycolysis as possible sites for carnosine's effect. Consecutive experiments by H. Oppermann showed a marked elevation of mRNA encoding pyruvate dehydrogenase kinase 4 (PDK4) in cells of human glioblastoma incubated with carnosine [8].

These findings instigated further investigations on carnosine's effect on PDK4 expression in human glioblastoma cell lines, which constitute a large part of this thesis. In addition, it was assessed, whether carnosine's effect on PDK4 transcription and on cell viability in general is limited to carnosine as a whole or can be mimicked by one of its two components L-histidine and  $\beta$ -alanine.

Part of the results presented in this work has previously been published as a joint work with H. Oppermann, J. Meixensberger and F. Gaunitz in Amino Acids [9].

## 1.2 Glioblastoma

Being the most frequent primary malignant tumour of the adult human brain and associated with a high mortality, glioblastoma poses a great challenge for researchers and clinicians in the fields of neurosurgery and neuro-oncology. It is categorized by the World Health Organization (WHO) as a glioma stage IV [10] and occurs with an incidence rate of 3.19 per 100,000 per year as observed by the Central Brain Tumor Registry of the United States [11]. The most common location for glioblastoma is the subcortical white matter of the cerebral hemispheres, but an occurrence in other parts of the CNS is not unheard of [10]. Apart from rare hereditary syndromes that are associated with an increased risk for glioblastoma such as Neurofibromatosis I, Turcot-Syndrome, Li-Fraumeni-Syndrome and multiple enchondromatosis [10], the tumour occurs spontaneously in most cases. Incidence in men is slightly higher compared to women [11]. Glioblastoma that develops out of previous lower grade lesions such as WHO grade II diffuse astrocytoma or WHO grade III anaplastic astrocytoma is termed

secondary glioblastoma, as opposed to primary glioblastoma, which arises de novo [10]. Secondary glioblastoma is less frequent and rather occurs in middle-aged patients, while primary glioblastoma is more common among patients over fifty years of age [12, 11].

On the cellular level, glioblastoma shows a very diverse picture of pleomorph cells with atypic nuclei. These can be recognized as astrocytic in some areas of the tumour, while being completely anaplastic in other regions [13]. Mitotic activity is generally high, and rapid microvascular proliferation can be found as well as areas of thrombosis or necrosis. The latter can usually be located in the centre of the tumour, sometimes presenting as cysts filled with liquefied necrotic tissue, or in band-like structures delineated by pseudopalisades of small glioma cells [10].

The growth characteristics of glioblastoma include rapid local growth as well as aggressive and diffuse invasiveness into adjacent parts of the brain. The invading cells often escape surgery and thus give rise to relapse tumours [10]. In combination, these factors lead to a survival rate of 35% for one year and 4.7% for five years after diagnosis [11]. In contrast to its extensive and fast spread throughout the brain, the tumour seldom infiltrates dura or bone. Metastases outside the CNS are very rare, be it by haematogenic, lymphogenic or cerebrospinal fluid paths [10]. Yet, a case of peritoneal metastasis via a shunting system has been reported [14].

Due to the fast development of the disease, which in extreme cases can be a matter of weeks only, and due to the fact that no predictive serum markers have yet been established in clinical routine, screenings for glioblastoma are limited to a small number of patients in whom occurrence of the disease is linked to a hereditary syndrome [15].

Several characteristic genetic alterations have been detected in glioblastoma cells, some of which allow a more accurate prognosis of survival time. Loss of heterozygosity at the long arm of chromosome 10 (LOH 10q) is commonly found in primary and secondary glioblastoma and correlates with a poor prognosis [12, 16]. Amplification and overexpression of endothelial growth factor receptor (EGFR) is also frequent [17], although mostly present in primary glioblastoma [18]. On the other hand, tumour protein 53 (TP53) mutation is suggested to be a sign of secondary development and indicates a better prognosis regarding survival time [19, 18]. Mutations of phosphatase and tensin homolog (PTEN) and deletion of p16<sup>INK4a</sup> can be found in 25% or respectively 31% of cases for primary glioblastoma, but are less frequent in the secondary type [12]. Rich *et al.* also detected a marked overexpression of the three genes Osteonectin, Semaphorin 3B and Doublecortin, which are involved in processes of cellular migration and seem to be linked to shorter survival [20].

Three molecular subclasses of glioblastoma termed proneural, proliferative and mesenchymal have been described by Phillips *et al.* [21]. While the proneural subclass expresses genetic markers related to neurons and neurogenesis like oligodendrocyte lineage transcription factor 2 (Olig2), brevican and delta-like 3 protein, the proliferative type shares patterns with haematopoietic stem cells and strongly

expresses markers of proliferation. Its characteristic markers are proliferating cell nuclear antigen (PCNA) and topoisomerase II  $\alpha$ . The mesenchymal type expresses markers that could be linked to dendritic cells and tissues of mesenchymal origin like muscle or bone, namely YKL-40, vimentin and CD44. Samples of this type also show a high expression of markers of angiogenesis like vascular endothelial growth factor (VEGF). Common features of the proliferative and mesenchymal subtype are a gain on chromosome 7 and a loss on chromosome 10 or 10q as well as a loss of the PTEN locus. Both of these subtypes appear to be correlated with a poorer prognosis, and tumours tend to shift towards the mesenchymal type upon recurrence [21].

A more recent classification by Verhaak *et al.* established four subtypes based on variations in expression and methylation status of PDGFRA, IDH1, Neurofibromin 1 and EGFR: The classical, mesenchymal, neural and proneural subtype [22]. Characteristic findings in the classical subtype include a gain on chromosome 7 and a loss on chromosome 10, together with a mutation or amplification of EGFR and a deletion of CDKN2A. Neuronal stem cell markers such as nestin are commonly found. The mesenchymal subtype again shows markers of mesenchymal tissue. Another distinct feature is a deletion or mutation of Neurofibromin 1 and a strong expression of genes related to tumour necrosis and the NF $\kappa$ B-pathway, which is in accordance with the frequent histological finding of necrosis in this subtype as reported earlier [21]. Characteristic patterns in the proneural type include mutations in IDH1 and mutation and amplification of PDGFRA. TP53 mutations are almost exclusively found in this subtype. Another feature, hinting at an oligodendroglial origin of tumours of this subtype, is a high expression of Olig2. The neural subtype is characterized by markers commonly found in neurons and normal brain tissue, like NEFL, SYT1, GABRA1 and SLC12A5. Patients with tumours of the proneural subtype showed a longer survival time in this study and were generally of a younger age compared to the other groups. The four subtypes also show a different susceptibility to standard methods of radiotherapy and chemotherapy treatment. The classical subtype responds especially well to treatment intensification while for the proneural subtype treatment intensity made no difference in survival time [22].

The current standard treatment for glioblastoma is comprised of surgical resection or biopsy in combination with adjuvant chemotherapy with the DNA-alkylating agent temozolomide and radiotherapy of the tumour site [15]. The treatment with temozolomide manages to elongate median survival time to 14.6 months compared to 12.1 months with radiotherapy alone and also improves progression free survival [23]. A study by Hegi *et al.* has shown that this benefit is more marked in patients with tumours carrying a methylation of the O6-methylguanine-DNA methyltransferase (MGMT) promoter [24].

Nitrosoureas present an alternative to temozolomide, although they are recommended as second line therapy [25, 15]. Local therapy with biodegradable 1,3-bis (2-chloroethyl)-1-nitrosourea (carmustine)

wafers combined with radiotherapy has proven to be more effective than radiotherapy alone for patients with malignant glioma in general, but this effect was only slight in patients with glioblastoma [26]. The rise of monoclonal antibodies as cancer therapeutics provided new ways of targeting glioblastoma, such as the humanized VEGF-antibody bevacizumab. As of now, bevacizumab is not part of the standard treatment for glioblastoma in the European Union, since phase II studies have shown that even though it manages to prolong progression free survival when added to a temozolomide and radiotherapy scheme, overall survival is not affected [27]. Nevertheless, it is frequently used for treating recurrent glioblastoma in other countries such as the United States, alongside other options of chemotherapy such as carboplatin [25].

To estimate survival for an individual patient, the genetic patterns described above are taken into consideration along with patient age and extent of necrosis, which, if high, are both associated with shorter survival [19, 28]. To detect recurrence of disease after resection, matrixmetalloproteinase-9 and YLK-40 can be used as serum markers [29]. Many efforts are still made to learn more about the mechanisms leading to the formation of the tumour and its progression and also to establish new, more effective ways of treatment or enhance the ones already in use.

### **1.3 Carnosine**

The dipeptide carnosine ( $\beta$ -alanyl-L-histidine) was first isolated from Liebig's meat extract by Gulewitsch and Amiradzibi in 1900 [30]. It is part of a family of histidine-containing dipeptides, among whose other members are anserin ( $\beta$ -alanyl-1-N-methylhistidine), ophidine ( $\beta$ -alanyl-3-N-methylhistidine), homoanserine ( $\gamma$ -aminobutyryl-1-N-methylhistidine), homocarnosine ( $\gamma$ -aminobutyrylhistidine) and their respective N-acetylated forms. Carnosine exists in two enantiomer forms, L-carnosine and D-carnosine. In this work, if not specifically mentioned otherwise, L-carnosine is meant when carnosine is mentioned.

The aforementioned histidine-containing dipeptides occur in high concentrations in excitable tissues of muscular and neural origin. Carnosine, anserine and in some cases ophidine are found in the muscular tissues of all vertebrates in varying contents, but in human muscle, only carnosine can be detected [31]. The carnosine content of human skeletal muscle reaches concentrations of up to 7.3  $\mu\text{mol/g}$  [32] or around 20 mmol/kg dry mass [33]. Its dependence on age, muscle fibre type and diet has been shown, and an influence of sex and exercise was also reported [34, 33], even though the latter two factors are still subject of ongoing discussion [35].

Tissue of the central nervous system of mammals is rich in carnosine as well, while anserine is only found in birds, but not in mammals [36, 37]. Especially high concentrations of carnosine and related immunoreactivity have been detected in neurons of the olfactory bulb [38–41] and there is evidence for axonal transport of carnosine from peripheral olfactory neurons into the olfactory bulb in rodents

[40]. Yet, carnosine is also present in other parts of the brain [39, 37, 42], where it is linked to cerebellar glial cells, ependymal cells and astrocytes [37, 43, 36]. In the human retina, only homocarnosine could be detected, which is conflicting with earlier findings of high retinal carnosine concentrations in frogs [44].

While some authors limit the occurrence of carnosine in humans to skeletal muscle and CNS tissue alone [45], Flancbaum *et al.* also located the dipeptide in stomach, kidney and various other tissue samples by using HPLC. They argue that it might act as a histidine reservoir for histamine synthesis in various tissues under conditions of stress [46].

Carnosine metabolism is conducted by a set of rather specialized enzymes, a fact that hints at the presumably high physiological relevance of the dipeptide. Carnosine is synthesized from  $\beta$ -alanine and L-histidine by the enzyme carnosine synthase (EC 6.3.2.11), which also synthesizes homocarnosine using  $\gamma$ -aminobutyrate and L-histidine at a lower rate of efficiency. It is thought to be identical with ATP-grasp domain containing protein-1 (ATPGDP1) [47]. The enzyme is found in skeletal muscle, in the olfactory bulb and in oligodendrocytes, the latter showing especially high rates of synthesis when fully differentiated [48–50]. An *in vitro* synthesis of carnosine could also be shown in rat C-6 glioma cells [42]. At least for the synthesis of carnosine in muscle, supply of  $\beta$ -alanine seems to be the limiting factor, as a study in horses by Dunnett *et al.* has shown [51].

The cellular uptake of carnosine can be achieved by four different transporters. The first two, oligopeptide transporter 1 and 2 (PEPT1 and PEPT2) belong to the SLC15 proton oligopeptide cotransporter family and are able to transport basically all dipeptides [52]. Only PEPT2 can be found in cells of the CNS, namely astrocytes, ependymal cells and cells of the choroid plexus [53]. The latter two transporters, peptide/histidine transporter 1 and 2 (PHT1 and PHT 2), have a much more narrow specificity and evidence for brain localization has been found for both [54, 55].

Two enzymes have been identified as being responsible for degrading carnosine in the human metabolism and splitting the dipeptide into its two components  $\beta$ -alanine and L-histidine. Termed carnosinases CN1 and CN2, they belong to the family of M20-metalloproteases or, more precisely, metal ion-dependent dipeptidases [56–59]. The CN1 type (EC 3.4.13.20) shows a substrate specificity for Xaa-His dipeptides, including carnosine, anserine and homocarnosine [58, 59]. Since this enzyme is secreted into the extracellular matrix and not present in the cytoplasm, it is also called serum carnosinase. Especially high concentrations are found in the brain [59].

In comparison, CN2, also termed tissue carnosinase (EC 3.4.13.3) for its exclusively intracellular localization, metabolises a much wider range of dipeptide substrates [57] and has thus been identified as a cytosolic nonspecific dipeptidase [60, 56]. CN2 is found in many tissues of the human body [57] and can be inhibited by bestatin [61]. Reduced levels of CN1 protein have been detected in serum of glioblastoma patients [62] and reduced activity of the enzyme was found in patients with mixed

dementia [63], multiple sclerosis, Parkinson's disease, and patients after cerebrovascular accident [64]. An age-related increase in activity of CN1 in the brain was shown by Bellia *et al.* and linked to increased oxidative stress due to a lack of carnosine as a buffer [65].

Various positive effects and capacities have been attributed to carnosine. Since McFarland and Holliday showed that carnosine can slow down senescence and even phenotypically rejuvenate cells in cultures of human diploid fibroblasts [66], the dipeptide has been regarded as a potential anti-senescent agent. As for the mechanisms by which this effect is achieved, anti-oxidant and radical scavenging capacities have been proposed and demonstrated [67–69]. Carnosine's ability to act as a metal chelator is another possible protective function [70, 71]. Furthermore, anti-glycating activities of the dipeptide have been proposed as a possible explanation, since this would prevent the formation of carbonyl groups in proteins, which is regarded as a process of cellular ageing [72, 73].

Apart from anti-senescence, modulatory effects on B- and T-cells [74] and a promotion of wound healing by carnosine [75] have been reported. In muscles, carnosine is thought to act as a pH-buffer during periods of exercise [33]. Some researchers have proposed that carnosine might function as a storage or transport vehicle for histidine and consequently play an important role in the synthesis of histamine, which can act either as a neurotransmitter [76] or as a mediator of inflammation [46].

With regard to processes in the CNS, studies have shown beneficial effects of carnosine on a broad spectrum of diseases. Neuroprotection by carnosine could be demonstrated for animal models of spinal cord injury [77], perinatal brain hypoxia [78], prion diseases [79], ischemic stroke [80] and mitochondrial dysfunction in Alzheimer's disease [81]. Most authors attributed these effects to antioxidative and metal chelating properties of the dipeptide. A diet supplemented with L-carnosine appeared to improve speech performance and other social and communicative skills of children with autism spectrum disorders [82]. As a side aspect, this result hints at the ability of orally administered carnosine to cross the blood brain barrier and reach effective concentrations in human brain tissue.

For this work, interactions of carnosine with neoplastic cells, their growth and their metabolism are especially of interest. Such interactions have been demonstrated *in vitro* and *in vivo*, with carnosine inhibiting growth and reducing viability of neoplastic cells in various experimental settings. Nagai and Suda first observed antineoplastic effects of carnosine in a mouse model of Sarcoma-180 cells, where carnosine inhibited growth and progression of the tumour, prolonged survival and reduced mortality [1]. Similar positive results could later be achieved in mouse models with human colon cancer cells [83] and Her2/neu-positive NIH3T3-fibroblasts [2]. In both studies, the oral or intraperitoneal application of carnosine significantly slowed down tumour growth, although complete growth inhibition, as reported by Nagai and Suda, could not be confirmed. Furthermore, the anti-neoplastic effect of carnosine was confirmed in a variety of different tumour entities and transformed cells assessed *in vitro*. Cells which were used in those studies include KRAS-mutated colon cancer cells [6],

PC-12 pheochromocytoma cells [5], cells isolated from human glioblastoma [3], and cells from the lines MRC-5V1 and MRC-5V2 (transformed human fibroblasts), as well as HeLa (human cervical carcinoma), A549 (human lung carcinoma), TE85 (human osteogenic sarcoma), BL-17/23 $\alpha$  (human bladder carcinoma), PC3 (human prostate carcinoma), CHO K1 (transformed hamster ovary fibroblasts) and WEHI 164 (mouse fibrosarcoma) cells [4]. All of these showed an anti-proliferative effect of carnosine on the neoplastic cells.

A lower rate of DNA synthesis [3], cell cycle arrest [5] and a decrease in formation of reactive oxygen species [6] are among the mechanisms detected by these experiments. In a later study, an interaction of carnosine with peptides involved in protein folding and HIF-1 $\alpha$ -signalling was shown for human glioblastoma cells [84]. Furthermore, both Renner *et al.* and Iovine *et al.* observed a decrease in ATP produced in tumour cells incubated with carnosine, consequently suggesting an interaction with processes of glycolysis [6, 7, 3]. As another link to glycolysis, Holliday and McFarland demonstrated that the limiting effect of carnosine on growth of tumour cells can be inhibited by pyruvate [4]. This antineoplastic activity of carnosine seems to stand in contrast to the positive effects on viability, proliferation and longevity of non-neoplastic cells mentioned above. A study in yeast cells by Cartwright *et al.* offers a possible explanation for this phenomenon, as it could show carnosine's anti-proliferative activity to be restricted to those yeast cells using an anaerobic metabolism [85]. In light of these findings, it was suggested that the shift of metabolism in tumour cells towards glycolysis even in an oxygenated environment could be the reason for the divergent effects of carnosine on neoplastic and healthy cells and tissues [86]. The high rate of glycolysis followed by lactic acid formation in the presence of oxygen is termed "Warburg Effect" in memory of Otto Warburg, who first described it [87]. In addition to that, these results again point towards a role of carnosine in processes of glycolysis in tumour cells. To further address this issue, Oppermann and Gaunitz performed qRT-PCR assays for a broad spectrum of enzymes involved in glycolysis using mRNA from human glioblastoma cells incubated with carnosine. In these experiments, a marked increase in mRNA copy number was shown for pyruvate dehydrogenase kinase 4 (PDK4) [9, 8]. This finding led to a more detailed analysis of carnosine's effect on the expression of PDK4 in human glioblastoma cells, which constitutes a large part of this thesis.

## 1.4 Histidine

Histidine is an aromatic amino acid characterized by an imidazole structure. Of the two enantiomers L-histidine and D-histidine, L-histidine is the physiologically more relevant form. Histidine is the precursor of histamine, which is synthesized from histidine by the enzyme histidine decarboxylase (EC 4.1.1.22) and – among other functions – also acts as a neurotransmitter and mediator of inflammatory reaction. Furthermore, histidine is also a constituent of carnosine and other dipeptides of the family of histidine containing dipeptides. Degradation of histidine to ammonia and urocanic acid is accomplished by the enzyme histidine ammonia lyase (EC 4.3.1.3). Histidine is considered an essential amino acid for humans [88], with symptoms of depletion including rash, neurological symptoms and hypalbuminaemia. Long-term toxicity studies in rats showed, after initial evidence for a higher frequency of sperm granulomas, no significant carcinogenic effect of the amino acid [89, 90]. In contrast, toxicity of histidine in cell cultures has been reported [91], which was linked to its ability to enhance DNA double strand breaking properties of hydrogen peroxide [92]. Histidine also potentiated oxidative toxicity of xanthine oxidase, bleomycin and  $\gamma$ -radiation on lymphocytes [93]. Yet, protection by histidine against inflammatory processes mediated by hydrogen peroxide or TNF- $\alpha$  has also been demonstrated [94].

Similarities, but also differences of the effects of L-histidine and carnosine have been pointed out by different groups of researchers. Son *et al.* demonstrated that both agents inhibit IL-8 secretion in Caco-2 cells under the influence of TNF- $\alpha$  or hydrogen peroxide, while L-histidine was more effective than carnosine. Notably, free histidine and dipeptides from which histidine could be cleaved within the cells were able to lower IL-8-mRNA copy number as well. Carnosine on the other hand did not influence IL-8-mRNA, and it was shown that carnosine remained uncleaved in the Caco-2 cells [95, 94]. This suggests that release of histidine might be the factor enabling carnosine to act on a transcriptional level. Hobart *et al.* also reported histidine to act similarly and more effectively than carnosine as an anti-crosslinking agent, using a model for the formation of  $\beta$ -amyloid plaques *in vitro*, and they suggested that histidine might be the active component of carnosine in this respect [96].

In experiments in yeast undertaken by Cartwright *et al.*, carnosine exerted a growth inhibitory influence on yeast, but this result could not be reproduced with L-histidine [85]. In contrast to this, Holliday and McFarland could show in their experiments on carnosine's anti-proliferative activity in transformed and neoplastic cells that a toxic effect similar to that of carnosine could be achieved by histidine and the histidine-containing dipeptide anserine, but not by  $\beta$ -alanine [4].



## 1.5 Human pyruvate dehydrogenase kinase 4 gene and enzyme

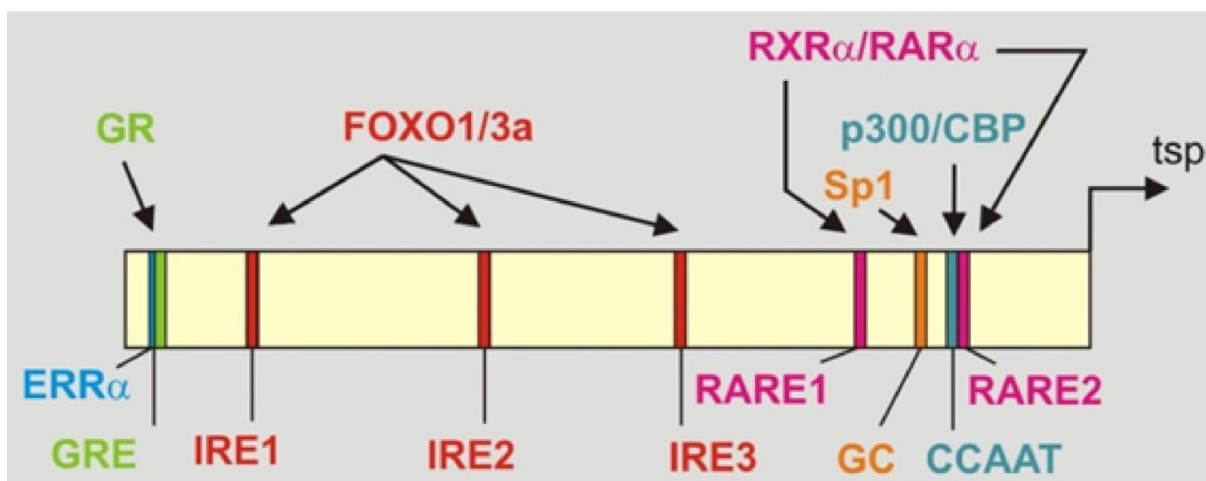
The enzyme pyruvate dehydrogenase kinase 4 (PDK4) is a member of a family of four protein kinases located in the mitochondrial matrix, whose function it is to phosphorylate and thus inhibit the pyruvate dehydrogenase complex (PDC). The enzyme binds to the E1 and E2 subunit of the PDC, the phosphorylation site being the E1 $\alpha$  component [97]. As the PDC is inactivated, less pyruvate is converted into acetyl-CoA by oxidative decarboxylation and consequently less acetyl-CoA is available for oxidation and production of ATP in the mitochondria. This mechanism is of importance for adapting the organism to fasting conditions by saving glucose in those tissues where other sources of energy, namely fatty acids, are available. The activity of PDK enzymes is inhibited by ADP, non-acetylated Coenzyme A, NAD<sup>+</sup> and pyruvate, while being stimulated by acetyl-CoA [98, 99].

The PDK4 gene, located on chromosome 7q21.3, was first characterized by Rowles *et al.* in 1996. Its transcript is detectable in various human tissues, the highest concentrations being found in skeletal and heart muscle [100]. PDK4-mRNA expression is known to be up-regulated in skeletal muscle, heart, kidney and liver of fasting and diabetic mammals [101–103] and further increased in human skeletal muscle following re-feeding after a fasting period [104]. In the rat brain, mRNA levels are higher under fasting conditions, while protein amounts remain unchanged [103]. Tsintzas *et al.* reported a down-regulation of PDK4-mRNA in human skeletal muscle under conditions of hyperinsulinaemia, which can be attenuated by free fatty acids [105]. Acute exercise increases PDK4 transcription and mRNA levels in skeletal muscle of humans, an effect which even lasts within a four hour recovery period [106]. This increase is dose-dependent and accompanied by hypomethylation of the PDK4 promoter [107]. In a study with obese and normal weight humans by Barres *et al.*, body mass index and insulin plasma levels appeared to be positively correlated with PDK4 promoter methylation levels and thus suppression of the gene, yet the pathological state of high methylation could be reversed by weight loss after gastric bypass surgery [108]. Elevated levels of PDK4 protein could be detected in the hearts of rats on a high-fat diet or in a state of hyperthyroidism [109].

Regarding the pathways by which these changes are mediated, several factors that regulate PDK4 gene transcription and mRNA expression have been identified and some binding sites for transcription factors within the promoter region are known. Very detailed reviews of these pathways have been published by Jeong *et al.* [110] and by Kwon and Harris [111]. Among those agents stimulating transcription of human PDK4 are glucocorticoids like dexamethasone, as shown by Kwon *et al.* who described a glucocorticoid response element (GRE) for the glucocorticoid receptor [112]. For this pathway to be effective, binding of forkhead transcription factors FOXO1a and FOXO3a is necessary. It takes place at three insulin responsive elements (IRE) located in the promoter region near the GRE. Interactions of GRE and FOXO factors with p300/CREB binding protein appear to be involved in this

regulation as well [112]. This stimulation is inhibited by insulin, which activates phosphorylation of the FOXOs by Protein Kinase B and thus disrupts their interaction with p300/CREB [112]. In human embryonic kidney cells of the HEK 293 line, transcription of PDK4 is up-regulated via peroxisome proliferator-activated receptors  $\beta/\delta$  (PPAR  $\beta/\delta$ ) and to a lesser extent by PPAR  $\alpha$ , which bind in the region of the transcription starting site [113]. PPAR  $\alpha$ -mediated induction was also observed in human myocytes [114]. Binding sites for retinoic acids (retinoic acid responsive elements: RAREs), which mediate enhanced expression when retinoic acid receptor  $\alpha$  or retinoic X receptor  $\alpha$  are bound, have also been identified in the proximal promoter of the human PDK4 gene [115]. Facilitation of transcription by Trichostatin A, a histone deacetylase inhibitor, has been described in the same study [115].

Factors that have been demonstrated to decrease PDK4 expression besides insulin are Erk and its activator EGF, as shown by Grassian *et al.* in three human mammary epithelial cell lines [116]. In addition, the human PDK4 promoter region contains possible binding sites for specificity protein 1 (SP1) and activating protein 2 (AP2), as well as a TATA-box like motif and a reverse CCAAT-motif [100]. An overview of the known regulatory elements in the human PDK4 promoter is shown in Figure 1.



**Figure 1: Regulatory elements in the human PDK4 promoter (Gaunitz 2014)**

Depicted are known regulatory elements upstream from the transcriptional start point (tsp) and transcription factors known to interact with these elements. Binding sites: ERR $\alpha$ : estrogen-related receptor response element; GRE: glucocorticoid response element; IRE: insulin response elements; RARE1/2: retinoic acid response elements 1/2; GC: GC box (SP1 binding site); CCAAT: p300/CBP binding site. Factors: GR: glucocorticoid receptor; FOXO 1/3a: forkhead-box-protein O 1/3a; RXR $\alpha$ /RAR $\alpha$ : retinoid/retinoic receptors; SP1: specificity protein 1; p300/CBP: p300/cAMP response element-binding protein-binding protein.

Further mechanisms of transcriptional regulation have been described in rodents. In the promoter region of the rat PDK4 gene, two thyroid hormone responsive element have been found, their activation leading to an increase of transcription, if peroxisome proliferator-activated receptor  $\gamma$  co-activator 1 $\alpha$  (PGC-1 $\alpha$ ) and CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ) are present as co-factors at their respective binding sites [117, 118]. Estrogen related receptors  $\alpha$  and  $\gamma$  (ERR  $\alpha$  and  $\gamma$ ) also lead to an activation of transcription in mice and rats, which is dependent on PGC-1 $\alpha$  and enhanced by FOXO1 activity [119, 120], and again this effect is inhibited by insulin [120]. Another way of transcriptional enhancement, which might be involved in PPAR-activation, was described for AMP-activated protein kinase (AMPK) interacting with fatty acids in rat cells [121]. In adipose tissue and hepatocytes of mice, PDK4-mRNA abundance is also increased under the influence of growth hormone, an effect mediated by phosphorylation of signal transducer and activator of transcription 5 (STAT5) [122, 123]. Jeong *et al.* also reported the identification of an inductive binding site for Krüppel like factor 15 [110].

As for regulation by miRNA, miR-107 has been named as a possible regulator of PDK4 expression in mice, its effect being a lack of increase in PDK4 protein levels despite a higher abundance of PDK4-mRNA in response to exercise [124]. Database research by Wilfred *et al.* strongly predicts a recognition sequence for miR-103/107 in the PDK4 3'UTR [125].

The role of PDK4 in cancer cells has not yet been assessed in depth. With regard to the Warburg effect, the PDKs in general appear to be promising targets [126], as their inhibition may prevent cancer cells from sustaining the typical anaerobic metabolism necessary for their growth and survival. Yet research has so far mostly focused on the inhibition of PDK1 [127],[97].

Interestingly, Grassian *et al.* reported that PDK4-mRNA, but not the mRNAs of the other three PDKs, is increased in cells detached from the extra cellular matrix, a state commonly linked to metastatic activity of tumour cells. Yet, in the same study, PDK4-overexpression could be linked to a slower proliferation and even death of matrix detached cells, and it could be shown by database queries that PDK4-mRNA expression is lower in tumour cells in comparison with their tissues of origin. The authors thus suggested that a high expression of PDK4, in contrast to the other PDK enzymes, might in fact have an anti-proliferative effect in tumour cells [116].

## **2 Objectives of the study**

This study investigates whether the effects of carnosine on cell viability and gene expression in human glioblastoma cells can be mimicked by and attributed to its component L-histidine. To answer this question, cell based assays measuring ATP production, dehydrogenase activity and lactate dehydrogenase release are employed. Furthermore, the expression of mRNA for the two carnosinase enzymes CN1 and CN2 is measured in cDNA from glioblastoma and normal brain tissue cells by qRT-PCR in order to determine whether these cells express the enzymes necessary to cleave L-histidine from carnosine in the first place.

Transcriptional effects on PDK4 that has been shown to be increased in its mRNA expression by carnosine are assessed by qRT-PCR and by using a reporter gene assay of the human PDK4 promoter and the 5' untranslated region (UTR) up to ~4000 bp upstream from the transcriptional start point (tsp). Here, again, it is assessed whether the effect of carnosine is mimicked by L-histidine and whether the effect can be linked to the PDK4 promoter region.

## 3 Materials and Methods

### 3.1 Materials

#### 3.1.1 Cell lines

U87	ATCC, Manassas, USA
T98G	ATCC, Manassas, USA
LN405	DMSZ, Braunschweig, Germany
HEK 293	ATCC, Manassas, USA
DH5 $\alpha$ T1	Life Technologies, Carlsbad, USA

#### 3.1.2 Primers

CNDP1 forward primer: 5' GAAGAATACCGGAATAGCAG 3'	biomers, Ulm, Germany
CNDP1 reverse primer: 5' CGGCCAGGTATGACTGTT 3'	biomers, Ulm, Germany
CNDP2 forward primer: 5' AGAAGCCCTGCATCACCTAC 3'	biomers, Ulm, Germany
CNDP2 reverse primer: 5' CCACCAAAGAGCCCATC 3'	biomers, Ulm, Germany
PDK4_660_BHI: 5' TTCAGGGATCCTGTTACGGGC 3'	biomers, Ulm, Germany
SP_GaulIII_HIII+26: 5' AGAATTCGTCATCGCTGAA 3'	biomers, Ulm, Germany
SP_GaulIII_46_NcoI: 5' CCCCTCAAGCCAGCCACAGT 3'	biomers, Ulm, Germany
hPDK4_up: 5' CATGGCGGGATCCTTTCTTATGGG CTG C 3'	biomers, Ulm, Germany
hPDK4_down: 5' CGCCTCCATGGTGACGCCACCC 3'	biomers, Ulm, Germany
PDK4 forward primer: 5' CTGTGATGGATAATTCCC 3'	biomers, Ulm, Germany
PDK4 reverse primer: 5' GCCTTTAAGTAGATGATAGCA 3'	biomers, Ulm, Germany
TBP forward primer: 5' TTGACCTAAAGACCATTGCAC 3'	biomers, Ulm, Germany
TBP reverse primer: 5' GCTCTGACTTTAGCACCTGT 3'	biomers, Ulm, Germany

### 3.1.3 Plasmids

pT81	S.K. Nordeen [128]
pCMV_hGLuc	P.J.K. GmbH, Kleinblittersdorf, Germany
p_NEGFP	Clontech Laboratories, Mountain View, USA
pUC19	Thermo Fisher Scientific, Waltham, USA
pex_GaulIII	Eurofins Genomics GmbH, Ebersberg, Germany

### 3.1.4 cDNA of normal brain tissue

Frontal Lobe	BioCat, Heidelberg, Germany
Occipital Lobe	BioCat, Heidelberg, Germany
Parietal Lobe	BioCat, Heidelberg, Germany
Temporal Lobe	BioCat, Heidelberg, Germany

### 3.1.5 Solutions and buffers

Coelenterazine Stock solution (100x)	1 mg coelenterazine 2.36 ml methanol
Gaussia buffer (1x, pH 7.8)	20 mM MOPS 75 mM KBr 1 mM EDTA 5 mM MgCl <sub>2</sub> 5 mM CaCl <sub>2</sub>
Gaussia Assay Reagent	10 µl coelenterazine stock solution 1 ml 1x Gaussia buffer
Hanks – Ca <sup>2+</sup> /-Mg <sup>2+</sup>	8.06 g NaCl 0.4 g KCl 0.06 g Na <sub>2</sub> HPO <sub>4</sub> x 2 H <sub>2</sub> O 0.06 g KH <sub>2</sub> PO <sub>4</sub> 0.48 g HEPES Adjust with NaOH to pH 7.4 add aqua dest. ad 1 l
L-carnosine stock solution	1 M L-carnosine in 0.7% NaCl
TAE buffer (+)	40 ml 25x TAE 80 µl EtBr add aqua dest. ad 2 l
TAE-Puffer (-)	20 ml 25 x TAE add aqua dest. ad 1 l
TAE-Puffer (25x)	900 ml aqua dest. 242 g Tris

### 3.1.6 Enzymes and kits

Accutase	PAA Laboratories GmbH, Pasching, Germany
Alkaline phosphatase from calf intestine	Roche Diagnostics GmbH, Mannheim, Germany
<i>Asp718I</i>	Roche Diagnostics GmbH, Mannheim, Germany
<i>Bam</i> HI Fast digest	Thermo Fisher Scientific, Waltham, USA
<i>Bgl</i> II Fast digest	Thermo Fisher Scientific, Waltham, USA
CellTiter-Blue™ Cell Viability Assay	Promega GmbH, Madison, WI, USA
CellTiter-Glo™ Luminescent Cell Viability Assay	Promega GmbH, Madison, WI, USA
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	Promega GmbH, Madison, WI, USA
<i>Eco</i> RI Fast digest	Thermo Fisher Scientific, Waltham, USA
<i>Hind</i> III	Roche Diagnostics GmbH, Mannheim, Germany
ImProm-II™ Reverse Transcription System	Promega GmbH, Madison, USA
<i>Kpn</i> I Fast digest	Thermo Fisher Scientific, Waltham, USA
MycoAlert™ Mycoplasma Detection Kit	Lonza Inc., Rockland, ME, USA
<i>Nco</i> I Fast digest	Thermo Fisher Scientific, Waltham, USA
<i>Pst</i> I Fast digest	Thermo Fisher Scientific, Waltham, USA
QIAprep® endofree Plasmid Maxi Kit	QIAGEN, Hilden, Germany
QIAprep® Plasmid Maxi Kit	QIAGEN, Hilden, Germany
QIAprep® RNeasy Minikit	QIAGEN, Hilden, Germany
QIAprep® Spin Miniprep Kit	QIAGEN, Hilden, Germany
QIAquick® Gel extraction kit	QIAGEN, Hilden, Germany
QIAquick® PCR purification kit	QIAGEN, Hilden, Germany
Rotor-Gene SYBR® Green PCR Kit	QIAGEN, Hilden, Germany

### 3.1.7 Media

LB-agar	1 l sterile H <sub>2</sub> O 25 capsules LB medium 15 g Selectagar Autoclave for 20 min at 125°C
LB-Medium	1 l sterile H <sub>2</sub> O 25 capsules LB medium Autoclave for 20 min at 125°C
Serum free medium	550 ml DMEM high glucose 4.5 g/l 5.5 ml/1% GlutaMAX <sup>TM</sup> 100 6 ml/0.1% Penicillin/ Streptomycin
SOB medium	10 g Casein Peptone 2.5 g yeast extract 0.25 g NaCl 5 ml 250 mM KCl adjust pH to 7.0 add aqua dest ad 500 ml
SOC medium	1ml SOB medium add MgCl <sub>2</sub> ad 10 mM add Glucose ad 20 mM
Standard medium for human glioblastoma cell lines	550 ml DMEM high glucose 4.5 g/l 55 ml/10% Fetal Bovine Serum Gold A15112-1936 5.5 ml/1% GlutaMAX <sup>TM</sup> 100 6 ml/0.1% Penicillin/ Streptomycin
TB-Medium	100 ml sterile solution of 17 mM KH <sub>2</sub> PO <sub>4</sub> 72 mM K <sub>2</sub> HPO <sub>4</sub> Add to 900 ml of autoclaved H <sub>2</sub> O solution containing 23 capsules of LB medium add 4 ml Glycerol
TfB I	10 mM Potassiumacetate 100 mM RbCl 10 mM CaCl <sub>2</sub> 50 mM MnCl <sub>2</sub> 15% Glycerol add aqua dest. ad 500 ml adjust to pH 5.8 with diluted acetic acid sterile filtration
TfB II	10 mM MOPS 10 mM RbCl 75 mM CaCl <sub>2</sub> 15% Glycerine add aqua dest. ad 100 ml adjust to pH 5.8 with 0.1 N KOH sterile filtration



### 3.1.8 Ready-made chemicals

1 kbp DNA Ladder	Promega GmbH, Madison, USA
10x dephosphorylation buffer	Roche Diagnostics GmbH, Mannheim, Germany
70% Ethanol	J.T. Baker, Deventer, Holland
Ampicillin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
$\beta$ -alanine	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Biozym Sieve Agarose 3:1	Biozym Scientific GmbH, Hess, Germany
Coelenterazin	P.J.K. GmbH, Kleinblittersdorf, Germany
DMEM high glucose 4.5 g/l, no pyruvate, no glutamate added, SKU-No. 11960-044	Life Technologies, Carlsbad, USA
Ethidiumbromid 1% (10 mg/ml)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Fast digest buffer	Thermo Fisher Scientific, Waltham, USA
Fetal Bovine Serum Gold A15112-1936	PAA Laboratories GmbH, Pasching, Germany
GlutaMAX™ 100x	Life Technologies, Carlsbad, USA
HCl	Carl Roth GmbH & Co KG, Karlsruhe, Germany
HEPES	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Isopropanol	Carl Roth GmbH & Co KG, Karlsruhe, Germany
K <sub>2</sub> HPO <sub>4</sub>	Carl Roth GmbH & Co KG, Karlsruhe, Germany
KBr	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
KCl	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
KH <sub>2</sub> PO <sub>4</sub>	Carl Roth GmbH & Co KG, Karlsruhe, Germany
KOH	Merck KGaA, Darmstadt, Germany
L-carnosine	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
L-histidine	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
LB Medium capsules	MP Biomedicals LLC, Solon, USA
Methanol	J.T. Baker, Deventer, Holland
MgCl <sub>2</sub>	Merck KGaA, Darmstadt, Germany
MOPS	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Mykocill AB	PAA Laboratories GmbH, Pasching, Germany
Na <sub>2</sub> HPO <sub>4</sub>	Carl Roth GmbH & Co KG, Karlsruhe, Germany

NaCl	Carl Roth GmbH & Co KG, Karlsruhe, Germany
NaOH	Carl Roth GmbH & Co KG, Karlsruhe, Germany
PBS buffer	PAA Laboratories GmbH, Pasching, Germany
Penicillin/ Streptomycin	PAA Laboratories GmbH, Pasching, Germany
Potassium acetate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
RbCl	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
SeaKern Agarose	Biozym Scientific GmbH, Hess, Germany
Select agar	Life Technologies, Carlsbad, USA
Trypan blue solution (0.4%)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Turbofect	Thermo Fisher Scientific, Waltham, USA

### 3.1.9 Instruments

-86°C Ultra-freezer MDF-U33V	Ewald Innovationstechnik GmbH, Bad Nenndorf, Germany
Accu-jet	Brand GmbH & Co KG, Wertheim, Germany
Autoclav Certo Clav	Ketomat Sterilizer-Division, Traun, Austria
BL 1500S scales	Sartorius AG, Göttingen, Germany
Centrifuge 5415D	Eppendorf AG, Hamburg, Germany
Centrifuge Beckman Avanti J-25	Beckman Coulter Inc. Fullerton, USA
Centrifuge 3K30 Sigma	Sartorius AG, Göttingen, Germany
Fixed-angle rotor 19776-H	Sartorius AG, Göttingen, Germany
Fridge and freezer	Liebherr-International Deutschland GmbH, Biberach an der Riss, Germany
Hera cell 150 Incubator	Thermo electron LED GmbH, Langenselbold, Germany
Labofuge UEC	UniEquip Laborgerätebau & Vertriebs GmbH, Martinsried, Germany
Mastercycler	Eppendorf AG, Hamburg, Germany
Mastercycler gradient	Eppendorf AG, Hamburg, Germany
Microscope Axiovert 135	Carl Zeiss AG, Feldbach, Switzerland
Microwave 1029 HGE	Privileg Quelle GmbH, Fürth, Germany
Mithras LB 940	Berthold detection systems GmbH, Pforzheim, Germany

Mupid exU submarine electrophoresis system	Advance Co. Ltd, Tokyo, Japan
NanoDrop 1000 Spectrophotometer	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Neubauer cell counting chamber	LO - Laboroptik GmbH, Friedrichsdorf, Germany
RotorGene 3000	QIAGEN, Hilden, Germany
Shaking incubator 4400™Innova	New Brunswick Scientific GmbH, Nürtingen, Germany
SpectraMax M5 Microplate Reader	Molecular Devices, Sunnyvale, USA
Sterile bench MSC Advantage	Thermo electron LED, GmbH, Langenselbold, Germany
Swingout-Rotor JS13.1	Beckman Coulter Inc. Fullerton, USA
Thermomixer comfort	Eppendorf AG, Hamburg, Germany
Transilluminator	Biostep Labor & Systemtechnik GmbH, Jahnsdorf, Germany
Unitherm water bath	UniEquip Laborgerätebau & Vertriebs GmbH, Martinsried, Germany
Universal 16 centrifuge	Hettich Zentrifugen GmbH & Co KG. Tuttlingen, Germany
Vortex 444-1372	VWR International
Vortex-Genie 2	Scientific Industries, Bohemia, USA

### **3.1.10 Software**

Argus X1 V.3.3.1	Biostep Labor & Systemtechnik GmbH, Jahnsdorf, Germany
Corel Draw X3	Corel Corporation, Ottawa, Kanada
Excel 2003®Microsoft	Microsoft Corporation, Redmond, USA
Microwin 2000	Mikrotek Laborsysteme GmbH, Overath, Germany
ND-1000 V3.1.2	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Origin pro 8.0	OriginLab Corporation, Northampton, USA
Rotor-Gene 6000 V 1.7	QIAGEN, Hilden, Germany
SoftMax Pro V 5.3	Molecular Devices, Sunnyvale, USA
Vector NTI Suite Version 6	Life Technologies, Carlsbad, USA

### 3.1.11 Consumables

0.2ml, 0.5ml, 1.5ml and 2ml microcentrifuge vials	Sarstedt AG & Co, Nümbrecht, Germany
6-well plates	TPP Techno Plastic Products AG, Trasadingen, Switzerland
Cell culture flasks 75 cm <sup>2</sup>	TPP Techno Plastic Products AG, Trasadingen, Switzerland
Cell culture petri dishes	TPP Techno Plastic Products AG, Trasadingen, Switzerland
Centrifuge vials 15ml	Sarstedt AG & Co, Nümbrecht, Germany
Cultube sterile culture tube, T406-2A	Simport, Beloeil, Canada
Falcon tubes 50ml, conical, flat-top	BD Biosciences, San Jose, USA
Filter Tips, various sizes	Greiner BIO-ONE GmbH, Frickenhausen, Germany
MultiGuard Barrier Tips, various sizes	Sorenson Bioscience, Inc., Salt Lake City, USA
Pasteurpipettes	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Peha soft powderfree gloves	Hartmann, Heidenheim, Germany
Petri dishes	Greiner BIO-ONE GmbH, Frickenhausen, Germany
PS-microplate 96 well, flat bottom (white, black, white/clear bottom, black/clear bottom)	Greiner BIO-ONE GmbH, Frickenhausen, Germany
Serological pipettes, various sizes	Sarstedt AG & Co, Nümbrecht, Germany

## **3.2 General microbiological and cytological methods**

### **3.2.1 Production of competent *E. coli***

In order to produce a large amount of a plasmid the plasmid has to be introduced into a bacterium. This process is called transformation and the bacteria capable of performing the uptake are referred to as competent cells.

For the experiments in this study, competent cells were produced by a chemical method using rubidium-chloride. In order to this, one colony of *E. coli* DH5  $\alpha$  T1 were transferred into 10 ml TB medium and incubated overnight at 37°C and a shaking frequency of 175 rpm. 5 ml of this culture were then transferred into 50 ml pre-warmed LB medium and further incubated for one hour at 37°C/ 175 rpm. Hereafter, 25 ml of this culture were transferred into 500 ml pre-warmed LB medium and again incubated at 37°C, this time at a shaking frequency of 300 rpm.

To quantify growth, “optical density” was measured at a wavelength of 550 nm in a 1 cm diameter cuvette using the Spectramax M5 microplate reader. Measurements were taken at 30, 60, 75 and 85 minutes after the initial measurement until growth and doubling speed presented a logarithmic curve. The whole culture was then divided into 50 ml aliquots, incubated on ice for 15 minutes and centrifuged for 5 minutes at 5000 rpm and 4°C. The resulting supernatant was removed completely and the pellets were resuspended in 5 ml ice-cold TFB I in a cold environment. In the following, the cells were incubated on ice for 15 minutes and then centrifuged at 3000 rpm and 4°C for 5 minutes. The supernatant was decanted and the pellets resuspended in 3 ml ice-cold TFB II. Meanwhile, the cells were kept on ice all the time. All resuspended cells were transferred into one vial, mixed and then divided into 200  $\mu$ l aliquots for freezing in liquid nitrogen. The frozen aliquots were stored at -80°C until use.

To test the transformation and colony forming competence of the bacteria produced, three 200  $\mu$ l aliquots were subjected to transformation with 1 pg, 10 pg and 100 pg of *pUC19* plasmid DNA as described in the transformation protocol below (see 3.2.2). The resulting colonies were counted, allowing an estimation of the amount of colonies formed per 1 pg of plasmid DNA.

### **3.2.2 Transformation of RbCl-competent *E. coli***

For transformation, 200  $\mu$ l of competent *E. coli* that had been produced using the rubidium-chloride method as described above and stored at -80°C, were incubated on ice for ten minutes. Then 8  $\mu$ l of water containing the DNA that was to be transferred into the bacteria were pipetted into the vials containing the bacteria. After another incubation period of 30 minutes on ice the bacteria were heated up to 42°C for 30 seconds in a block heater before being put back on ice for another 5 minutes. The bacteria were then transferred into culture vials containing 1 ml of SOC medium and incubated at 37°C for 2 hours in a shaking incubator at a shaking frequency of 225 rpm. Following this, the content of

each culture vial was transferred into a 2 ml micro centrifuge tube to be centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and the remaining pellet resuspended in 200 µl of SOB medium before being transferred and spread onto LB-agar plates containing 0.1 mg/ml ampicillin. These plates were then incubated overnight at 37°C.

### **3.2.3 Preparation of plasmid DNA from cultures of transformed *E. coli***

After transforming and culturing RbCl-competent *E. coli* as described in the previous paragraph, plasmid DNA preparation was performed to first determine which of the resulting colonies was carrying the required plasmid.

For DNA purification from colonies of transformed *E. coli*, single colonies were picked from the agar plates on which they had been incubated using a sterile wooden pick and transferred into culture vials containing 2 ml TB-medium with 0.1 mg/ml ampicillin. These cultures were then incubated overnight at 37°C in an incubator shaking at 175 rpm.

After incubation, 1 ml of each liquid culture was subjected to plasmid preparation using the QIAprep® Spin Miniprep Kit according to the manufacturer's protocol, while the remaining 1 ml of each culture was supplied with 1 ml of fresh TB-medium and 0.1 mg/ml ampicillin and stored at 4°C for further cultivation. The DNA retrieved from the preparation was digested by restriction enzymes and analysed by gel electrophoresis to assess whether the required plasmid was expressed by one of the colonies. Details on restriction enzymes, digestion and electrophoresis conditions used for the analysis of plasmids in this work can be found below (see 3.4).

If one of the liquid cultures was carrying the desired plasmid, it was transferred into 100 ml TB medium with 0.1 mg/ml ampicillin for overnight incubation at 37°C, shaking at 175 rpm. From this 100 ml culture, plasmid DNA was purified using the QIAprep® Plasmid Maxi Kit or QIAprep® endoFree Plasmid Maxi Kit according to the respective protocols provided by the manufacturer. The resulting pellet of purified DNA was resuspended in 400-600 µl TE-buffer and its DNA concentration was measured using the Nanodrop 1000 Spectrophotometer and the ND-1000 Software. In these measurements, the ratio of optical density at 260 nm and 280 nm was used as an indicator of DNA purity, its target value lying between 1.86 and 1.91 for DNA that was to be used in transfection experiments. Plasmids were then stored at 4°C until further use.

### **3.2.4 Cell culture conditions for human cell lines**

The human glioblastoma cell lines U87, T98G, LN405 and the human embryonic kidney cell line HEK293 were used for the experiments in this study. U87, HEK 293 and T98G were purchased from the ATCC (Manassas, USA), LN405 was obtained from the German collection of microorganisms and cell cultures of DMSZ, Braunschweig, Germany. All cell lines were checked for their genetic identity according to the recommendations of the ATCC® Standards Development Organization's standard for cell line

authentication (ANSI/ATCC ASN-0002-2011) [129]. Genotyping was done by Genolytic GmbH, Leipzig, Germany. For long-term storage of cells, a -196°C liquid nitrogen tank was used. Before being used in the experiments, the cells were defrosted and transferred into 75 cm<sup>2</sup> culture vials. After a resting period of 24 hours, the cells were seeded into new 75 cm<sup>2</sup> culture vials at a density of 1x10<sup>6</sup> cells per 10 ml medium.

The culture medium used for all cell lines, which will be referred to as “standard medium” in the following, was composed as stated in the Materials and Methods chapter (see 3.1.4). It has to be noted that during the conduct of this study different charges of FBS were used for preparing the cell culture medium. A quantitative, but not qualitative effect of these changes was suspected in some experiments, while at the same time the FBS manufacturer PAA, Cölbe, Germany, reported irregularities in FBS production [130]. Therefore, some experiments were repeated with varying charges of FBS to confirm that no qualitative differences in results occurred after changing FBS charges. Wherever possible, FBS from one single charge was used.

The cells were kept in an incubator at 37°C in an atmosphere of 5% CO<sub>2</sub>. Every three to four days, when confluence was reached, the cells were detached and split into fractions to be seeded into new vials. For detaching, the old medium was removed with a sterile glass pipette and replaced by 4 ml Hank's -/- buffer. After 4 minutes the buffer was removed and the cells were incubated with 2 ml Accutase at 37°C for 5 minutes. This incubation period could be prolonged slightly, if a microscopic overview did not exhibit a sufficient detachment of the cells from the flask. After all cells in the flask were detached, the enzymatic reaction of Accutase was blocked by adding 4 ml standard medium. A fraction of the cell suspension obtained in this way could then be seeded into a new flask for further cultivation and supplied with fresh medium. The exact size of each fraction depended on the speed of proliferation for the respective cell line.

If the cells were to be seeded for an experiment, the exact number of cells per ml suspension had to be assessed. In this case, the cell suspension was transferred into 50 ml centrifuge tubes and centrifuged for 5 minutes at 800 rpm. The supernatant was removed and the cell pellet was resuspended with 1 ml fresh medium per estimated 1x10<sup>6</sup> cells. 20 µl of this new suspension were mixed with 20 µl Trypan Blue Dye in a microcentrifuge tube and pipetted into a cell counting chamber (0.0025 mm<sup>2</sup>). After counting the non-dyed, vital cells in four big squares of the counting chamber under a microscope, the number of cells per ml suspension could be calculated.

### 3.3 Assay methods and protocols

#### 3.3.1 Transfections and reporter gene assays

The introduction of “naked” DNA into eukaryotic cells is termed transfection and can be achieved by chemical or physical means. As this procedure is quite stressful for the cells, careful selection of transfection conditions is indispensable. The transfection conditions used in the experiments for this study were established by a preliminary experiment aiming at optimizing the transfection conditions for cells of the U87 line. A description of this experiment can be found in the appendix of this work (see 8).

As a result, the following protocol was adopted for transfection: Cells grown to a confluence of about 80% in 75 cm<sup>2</sup> flasks were detached using Accutase and counted as previously described. They were then seeded into the wells of black 96-well Greiner microplates with a clear bottom in 200 µl of standard medium in each well at a density of 10,000 cells per well. Twenty-four hours later, the transfection mixture was prepared. For each well it contained 100 ng of the DNA that was to be transfected, 10 µl of serum free medium and 0.2 µl of the cationic polymer based transfection reagent Turbofect. After adding the transfection reagent by slow dripping, the mixture was vortexed and left to stand for 20 minutes. By adding standard medium, the mixture was then expanded to 200 µl for each well and the old medium on the cells was replaced by it. To differentiate general effects on expression from effects linked to the target gene sequence, cells were transfected with a control plasmid which contained only a basic promotor (*Herpes simplex* thymidine kinase promoter without enhancer elements). The cells were then incubated for 100 minutes at 37°C and in a 5% CO<sub>2</sub> atmosphere. Cell density and the amount of medium, DNA and the transfection agent Turbofect had to be adjusted to different sizes of plates for some of the experiments. For 10 cm diameter petri dishes, 1x10<sup>6</sup> cells were seeded into 10 ml medium and later transfected with 3.3 µg of DNA. Afterwards, the medium was exchanged for fresh medium, this time supplemented to establish the various conditions that were to be assessed in the respective experiments. The incubation time with a certain agent stated in the description of the individual experiments is thus always counted from this point of time.

The reporter gene containing the promoter and the 5'-UTR of the human PDK4 gene used in the experiments was encoding the luciferase of *Gaussia princeps*. This luciferase is secreted into the medium and can thus be measured at various points after transfection, since there is no need to lyse the cell for measurement. After the incubation time required for the respective experiments, 5 µl of medium from each well was pipetted onto a white 96-well Greiner microplate and left to adjust to room temperature for 20 minutes. The assay reagent was prepared using 10 µl coelenterazine stock solution per 1 ml 1x *Gaussia* buffer, each well receiving 50 µl of the reagent. Luminescence of each well was detected with a delay of 1.6 seconds after injection of the reagent at an integration time of 0.5 seconds. All *Gaussia* luminescence assays were performed using the Mithras LB 940 luminometer



and the Microwin 2000 software. The different conditions in the experiments were each applied to at least six wells of a 96-well microplate at a time, thus allowing a six-fold measurement for each condition.

### **3.3.2 mRNA-isolation and qRT-PCR**

For experiments investigating changes in mRNA expression, a three step procedure of mRNA-isolation, cDNA synthesis and qRT-PCR was performed.

In the beginning, cells grown close to confluence were detached from their culture flasks, counted and seeded onto petri dishes of 10 cm diameter at a density of  $1 \times 10^6$  cells per dish in 10 ml standard medium. Alternatively, 6-well dishes could be used, containing 2 ml of medium and 250,000 cells per well. The cells were left to settle for 24 hours in the incubator. After that period, medium was exchanged to supplemented medium or non-supplemented medium according to the conditions of interest for the experiment. Depending on the incubation periods that were to be assessed, mRNA was isolated for each condition and its respective control using the QIAprep® RNeasy Minikit according to protocol at various points in time. After each isolation procedure, the concentration of RNA was measured using the NanoDrop 1000 Spectrophotometer. The RNA was then stored at -80°C. Later, cDNA was synthesized by reverse transcription using 500 ng of RNA and the ImProm-II™ Reverse Transcription System according to protocol, employing random primers. The cycling sequence used consisted of the following steps: 5 minutes 25°C, 60 minutes 42°C, 15 minutes 70°C, then cooling to 4°C. Using the cDNA retrieved in this way, qRT-PCR was performed using a Rotor Gene 3000 cycling system with SYBR® Green as a marker substance. All measurements were done in triplicate or quadruplicate. For normalization, the TATA-box binding protein (TBP) gene was analysed at least in duplicate. The following primers were used for qRT-PCR (for sequences see 3.1.2): PDK4 forward and reverse primer (amplicon length: 148 bp, melting temperature: 81.1°C), TBP forward and reverse primer (amplicon length: 424 bp, melting temperature: 83.9°C), CNBP1 forward and reverse primer (amplicon length: 152 bp, melting temperature: 83.8°C) and CNBP2 forward and reverse primer (amplicon length: 150 bp, melting temperature: 86.4°C). The primers used for qRT-PCR were purchased from biomers, Ulm, Germany. Prior to use, all primers had been checked for matching melting temperatures, potential mispriming sites, self-complementarity and splicing variants by employing the NCBI/Primer-BLAST algorithm.

In measurements of CNBP1 and CNBP2 mRNA, cDNA from normal brain tissue was used for comparing it with cDNA from glioblastoma cell lines. This cDNA was purchased from BioCat, Heidelberg, Germany. Four samples originating from the frontal, occipital, parietal and temporal lobe of the brain were tested. The measurement results were analysed using the Rotor Gene 6 Software Version 6.1/Build 93. Melting curve analysis was used to check for misamplifications and copy number was calculated using

a standard curve established in measurements on standard plasmids purchased from Eurofins/mwg operon Genomics GmbH, Ebersberg, Germany. To detect changes in mRNA-expression, the copy number of the target mRNA was compared to the copy number of TBP mRNA in treated cells and the calculated ratio was compared to the corresponding ratio of untreated control cells. In case of low overall copy numbers, instead of this the  $C_t$  of the reference gene was subtracted from the  $C_t$  of the target gene and the negative binary logarithm of the resulting  $\Delta C_t$  was used to compare control and samples.

### **3.3.3 Cell viability assays**

For assessing cell viability, cells were seeded on black or white 96-well Greiner microplates with a clear bottom at a density of 5,000 or, in case of the relatively larger sized LN405, 2,500 cells per well in 200  $\mu$ l standard medium. The cells were left to incubate overnight and 24 hours later medium was exchanged to establish the conditions to be assessed in the respective experiments, the volume of medium in each well now being 100  $\mu$ l. Differences in dilution of medium were compensated by adding 0.7% NaCl solution. To allow for a six-fold measurement, a single condition was always applied to six wells each.

Cells were then again incubated and cell based assays were performed at such times as required for the experiments. For luminescence measurement, white plates were used and black plates for fluorescence, respectively. The plates were read at medium sensitivity using the SpectraMax M5 Microplate Reader and the Softmax Pro V 5.3 software.

Cell viability was assessed using the CellTiter-Glo™ Luminescent Cell Viability Assay (CTG) and the CellTiter-Blue™ Cell Viability Assay (CTB). The former one indicates ATP production by a luminescent reaction. To perform this assay, medium was removed from the cells and replaced with 50  $\mu$ l of fresh medium and 50  $\mu$ l of the CellTiter-Glo™ reagent. Prior to this, the reagent had been prepared by mixing 10 ml of CellTiter-Glo™ buffer with the CellTiter-Glo™ substrate. The plates were covered from light and left to stand on a shaking plate shaking at very low speed for 2 minutes, then left to settle for 10 minutes and finally, endpoint luminescence was measured.

The CellTiter-Blue™ assay indicates cell viability by dehydrogenase activity, the enzymatic reaction being coupled to the conversion of the redox dye resazurin into the fluorescent resorufin. The amount of resorufin produced is proportional to the number of viable cells on a 96-well plate. After replacing the medium in each well with 100  $\mu$ l of fresh medium and 20  $\mu$ l of the CellTiter-Blue™ reagent, the plates were left to incubate at 37°C and 5% CO<sub>2</sub> atmosphere for 3 hours. Then, fluorescence was measured at an excitation wave length of 560 nm and a detection wave length of 590 nm.

To evaluate cytotoxicity, the CytoTox-ONE™ homogenous membrane integrity assay (CTX) was used, which indicates release of lactate dehydrogenase (LDH) into the medium. LDH is released by cells with

a damaged membrane and thus indicates necrotic cell death. In this assay, again the enzymatic reaction is coupled to the conversion of resazurin into the fluorescent resorufin. Here, the resulting amount of resorufin is proportional to the number of necrotic cells. For the assay, 50 µl of medium from each well were pipetted onto a black 96-well Greiner microplate with a flat, black bottom and left to stand for 30 minutes at room temperature. After that, 50 µl of the reagent that had been prepared by adding 11 ml of buffer to the substrate mix, were pipetted into each well and incubated again at room temperature for another 10 minutes. Then, fluorescence was measured at 590 nm after excitation at 560 nm.

### 3.4 Construction of the reporter gene (-3968/+319)\_PDK4\_GaulIII

To investigate whether changes in the expression of a gene can be linked to a certain part of its sequence, reporter genes have to be constructed in which the sequence of interest controls transcription of a gene encoding a detectable product such as a luciferase - an enzyme catalysing a light emitting reaction. The sequence of interest and the sequence encoding the reporter enzyme are part of a plasmid vector that is transfected into cells in which expression should be investigated.

In this work, the vector *pT81* – which originally contained a firefly luciferase gene [128] – was used to create a vector containing the luciferase from *Gaussia princeps*, designated *pT81\_GaulIII*. Additionally, some restriction enzyme recognition sites were mutated to facilitate sub-cloning.

To obtain the reporter gene with the human PDK4 upstream region, a 4287 bp insert containing a sequence starting 3968 bp upstream from the transcription starting point of the human PDK4 gene and ending 319 bp downstream of it was obtained by PCR using genomic DNA of the cell line T98G and the following primers:

hPDK4\_up: 5' CATGGCG**GGATCC**TTTCTTATGGGCTGC 3'

hPDK4\_down: 5' CGCCT**CCATGG**TGACGCCACCC 3'

(Note: restriction enzyme recognition sequences are bold and underlined)

In the following step, the PCR product and the vector *pT81\_GaulIII* were digested with the restriction enzymes *Bam*HI and *Nco*I to remove the pT81 promoter and to generate phosphorylated “sticky” ends. The vector was dephosphorylated using alkaline phosphatase in order to prevent religation of the empty vector during ligation. After agarose gel electrophoresis of both, the digested and dephosphorylated vector and the digested PCR product, gel extraction was performed (QIAquick Gel Extraction Kit). Finally the PCR product and the vector were ligated using T4-DNA-ligase (Promega) to obtain the reporter gene (-3968/+319)\_PDK4\_GaulIII. To verify that the reporter gene which was finally

obtained after transformation of the ligation reaction into competent cells and colony screening carried the expected sequence, crucial parts of the reporter gene were sequenced using the following primers:

PDK4\_660\_BHI: 5' TTCAGGGATCCTGTTACGGGC 3'  
 SP\_GaulIII\_HIII+26: 5' AGAATTTCGTCATCGCTGAA 3'  
 SP\_GaulIII\_46\_NcoI: 5' CCCCTCAAGCCAGCCACAGT 3'

The final construct includes 3968 bp 5'-upstream from the transcriptional start point (tsp) and 319 bp downstream of it, including the transcriptional start codon that is now the start codon of the *Gaussia* luciferase. Within the first 850 bp upstream of the tsp, several elements known to be involved in transcriptional regulation of PDK4 are present [111, 100], an overview of which is depicted in Figure 1 in the introduction (see 1.5).

### 3.5 Statistical analysis

Statistical analysis was performed using the algorithms of Microsoft® Excel 2003. To calculate significance *Student's t test* was used. Results were regarded as significant at a value of  $p < 0.05$ . To compare relative changes from two experiments with different means and standard deviations, the overall mean and standard deviation was calculated using the following formula for Gaussian error propagation:

$$\frac{\Delta E}{\frac{a}{b}} = \sqrt{\left(\frac{\Delta a}{a}\right)^2 + \left(\frac{\Delta b}{b}\right)^2}$$

In this formula  $a$  represents the mean of data set 1 and  $b$  the mean of data set 2,  $\Delta a$  and  $\Delta b$  represent the respective standard deviations of the two data sets.  $\Delta E$  is the propagated error.

For determining the concentration, which results in a half-maximal response to a substance in the cells tested (EC50), the Boltzmann function was employed according to the algorithm implemented in the Origin 8 software. The function results in an approximated sigmoidal curve based on the measurement data, from which the EC50 can be calculated. In cases a fitting of the Boltzmann function was not possible, an exponential function calculated by the algorithm of the Origin 8 software was used.

## 4 Results

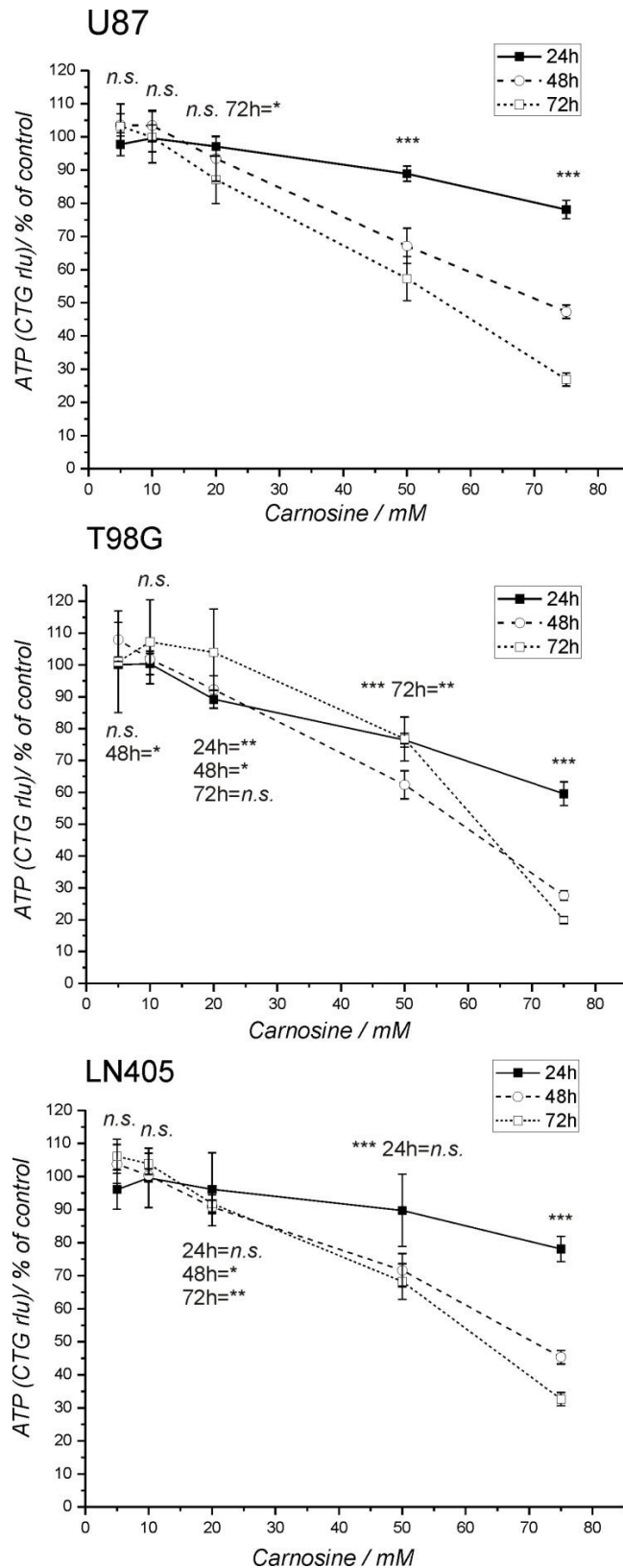
### 4.1 Cell viability of glioblastoma cells under the influence of carnosine, L-histidine and $\beta$ -alanine

Although the anti-neoplastic effect of carnosine has been confirmed by several groups, little is known on whether one of the amino acids that form the dipeptide has an anti-neoplastic effect by itself and may even be responsible for the anti-cancerous capacity of carnosine. In order to shed further light on this issue, cells from the human glioblastoma cell lines U87, T98G, and LN405 were cultivated in medium with varying concentrations of carnosine, L-histidine and  $\beta$ -alanine and cell viability was assessed by different cell based assays. The cells were seeded onto 96-well Greiner microplates, left to settle in standard medium for 24 hours and then incubated with fresh standard medium containing the supplements. The conditions assessed in the experiments included concentrations of 0, 10, 20, 50 or 75 mM carnosine, 0, 5, 10, 20, 30, 40 or 50 mM L-histidine and 20 or 50 mM  $\beta$ -alanine. It has to be taken into consideration that the medium used in this experiment already contained 0.2 mM L-histidine as stated by the manufacturer. After an incubation time of 24 hours, 48 hours and 72 hours CellTiter-Glo™, CellTiter-Blue™ and CytoTox-ONE™ assays were performed on one plate for each cell line. Six wells for each condition were measured in each assay. To allow a comparison of the results among the different experiments, all measured values were converted into relative changes compared to the respective untreated control. The experiment was repeated three times.

#### 4.1.1 ATP production under the influence of carnosine, L-histidine and $\beta$ -alanine

The negative effect of carnosine on production of ATP in human glioblastoma cells that has been previously shown by Renner *et al.* for T98G [7], could be confirmed for all three cell lines as depicted in Figure 2. The intensity and time of onset of this effect varied markedly among the cell lines, with T98G reacting with the strongest decrease in ATP levels and U87 and LN405 showing a weaker and later response. Concentrations of up to 10 mM lead to a small elevation of ATP levels compared to those cells receiving non-supplemented medium, but this was not significant except in T98G at 5 mM after 24 hours.

Beginning from 20 mM and upwards, ATP production was significantly lowered in all cell lines after 48 hours at the latest. After 72 hours at 75 mM, ATP levels had decreased to 26% ( $\pm$  2%) of the control in U87, 20% ( $\pm$  1%) in T98G and 32% ( $\pm$  2%) in LN405.

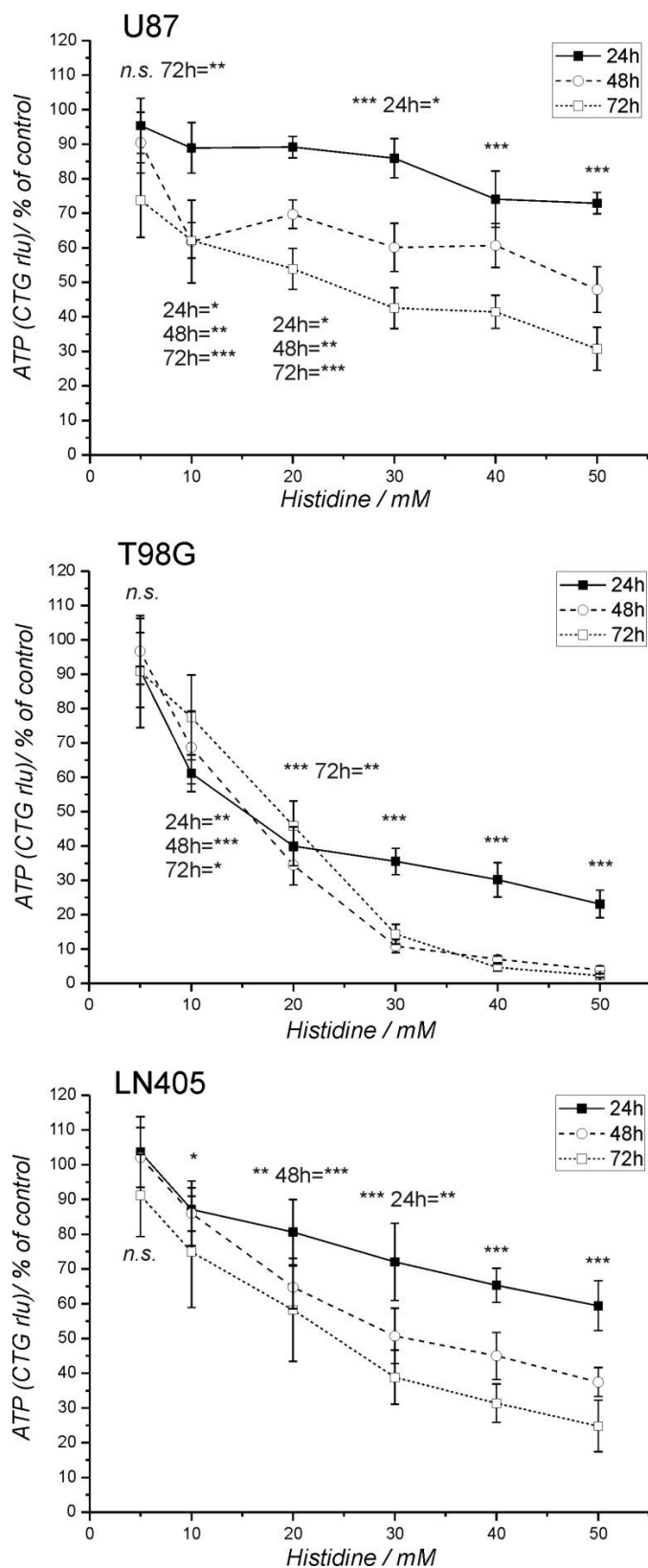


**Figure 2: ATP production after incubation with carnosine**

Depicted are ATP-totals of U87, T98G and LN405 cells after incubation with 0, 5, 10, 20, 50 and 75 mM of carnosine. Cells were incubated for 24, 48 and 72 hours. After the incubation the amount of ATP was determined employing the CellTiter-Glo™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significance as calculated by *Student's t-test* is marked by asterisks: \* =  $p < 5 \times 10^{-2}$ ; \*\* =  $p < 5 \times 10^{-3}$ ; \*\*\* =  $p < 5 \times 10^{-4}$ ; n.s. = not significant.

L-histidine elicited a similar negative effect on ATP production, which could be achieved by lesser concentrations as compared to carnosine (see Figure 3). Again, there were differences in the response of the three cell lines. A significant decline in ATP production could be detected in all lines starting from 10 mM L-histidine after 24 hours of incubation. This time again, T98G appeared to be most sensitive to L-histidine treatment, reacting with a drop of ATP levels to 61% ( $\pm$  5%) of control after 24 hours incubation with 10 mM L-histidine. In both U87 and LN405 the effect appeared later than in T98G, although here as well a significant reduction could be detected in comparison to the untreated control. After 72 hours of incubation with 50 mM L-histidine, the ATP levels of all cell lines were markedly below fifty percent of their respective controls.

In addition, a microscopic examination of the cells revealed that the number of cells was visibly decreased in those wells treated with more than 20 mM L-histidine and cells appeared to be less vital.



**Figure 3: ATP production after incubation with L-histidine**

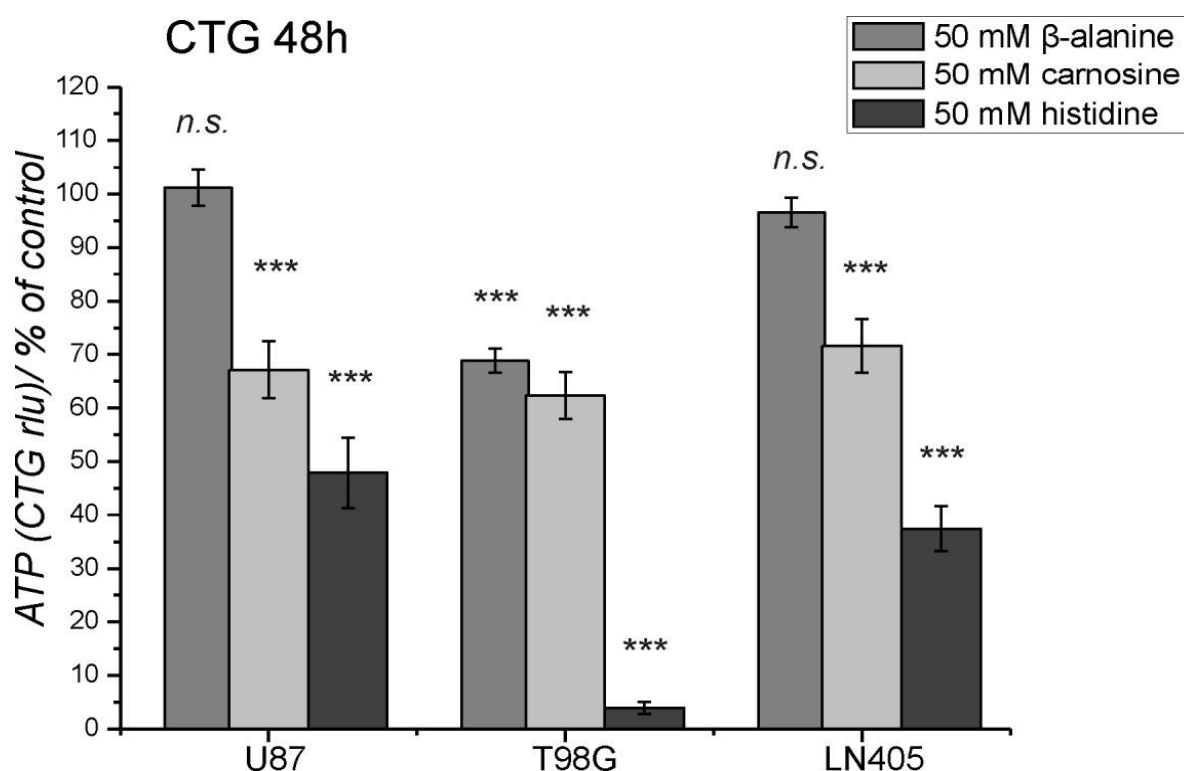
Depicted are ATP-totals of cells from the lines U87, T98G and LN405. Cells were incubated with 5, 10, 20, 30, 40 and 50 mM L-histidine for 24, 48 and 72 hours. Then ATP amounts were determined by CellTiter-Glo™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significance as calculated by *Student's t-test* is marked by asterisks: \* =  $p < 5 \times 10^{-2}$ ; \*\* =  $p < 5 \times 10^{-3}$ ; \*\*\* =  $p < 5 \times 10^{-4}$ ; n.s. = not significant.



A comparison of the effect of carnosine with the changes elicited by its components L-histidine and  $\beta$ -alanine is presented in Figure 4, which shows the relative decrease in ATP totals as detected by luminescence using the CellTiter-Glo™ assay. ATP totals were compared to the untreated control after incubation with 50 mM of each substance for 48 hours. Similar results could be detected for 20 mM concentrations of the substances and for 24 and 72 hours (data not shown). As can be seen,  $\beta$ -alanine treatment did not lead to a significant decline in ATP production in cells from the lines U87 and LN405, while carnosine could lower it significantly. Only in T98G, a reduction to 69% ( $\pm 2\%$ ) under the influence of  $\beta$ -alanine could be seen, with carnosine achieving a reduction of 62% ( $\pm 4\%$ ).

L-histidine on the other hand lead to a significant decrease of ATP levels in all cell lines, even drastically exceeding the effect of carnosine and  $\beta$ -alanine in T98G.

Therefore, it can be assumed, that the imidazole ring of L-histidine is pivotal for the effect of carnosine on ATP production.

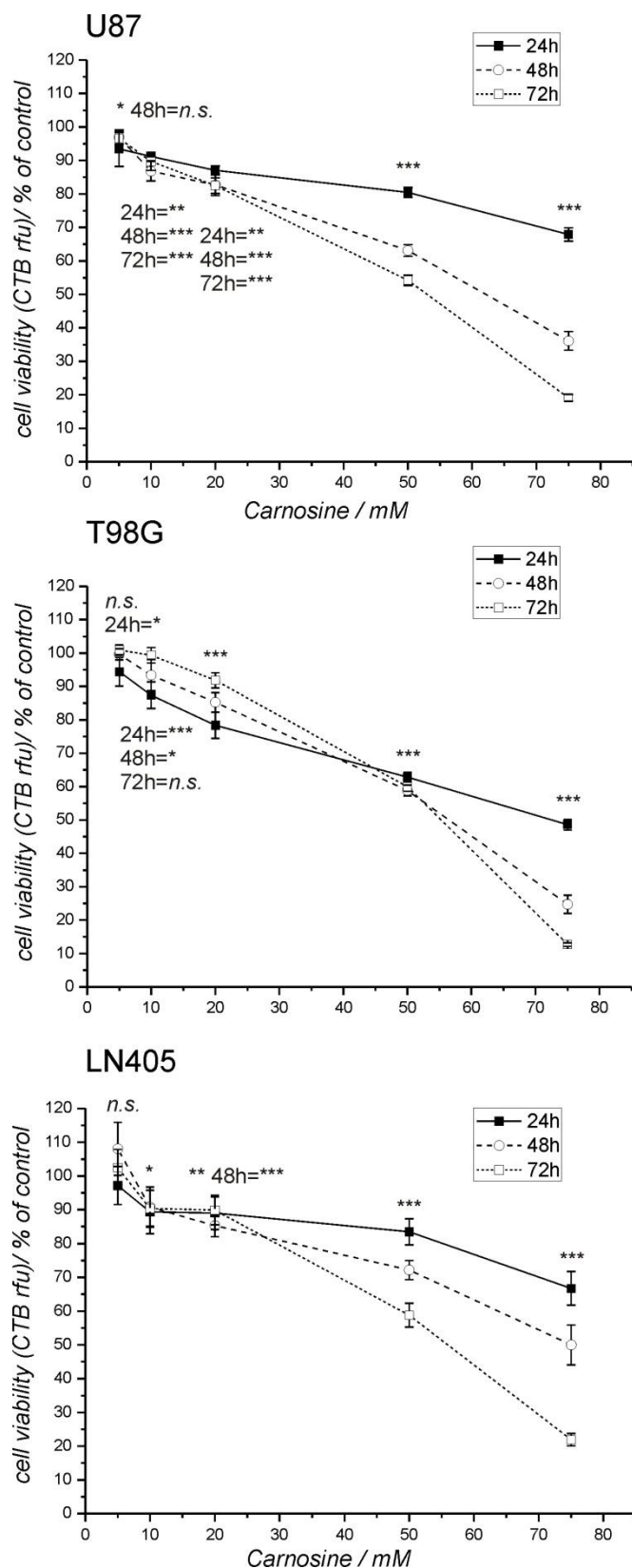


**Figure 4: ATP totals after 48 hours incubation with carnosine, L-histidine and  $\beta$ -alanine**

The figure shows ATP totals of cells from the lines U87, T98G and LN405. Cells were incubated with 50 mM of carnosine, L-histidine or  $\beta$ -alanine for 48 hours. Then ATP amounts were measured using the CellTiter-Glo™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significant differences to the respective controls as calculated by Student's *t*-test are marked by asterisks: \*\*\*=  $p < 5 \times 10^{-4}$ ; n.s.= not significant.

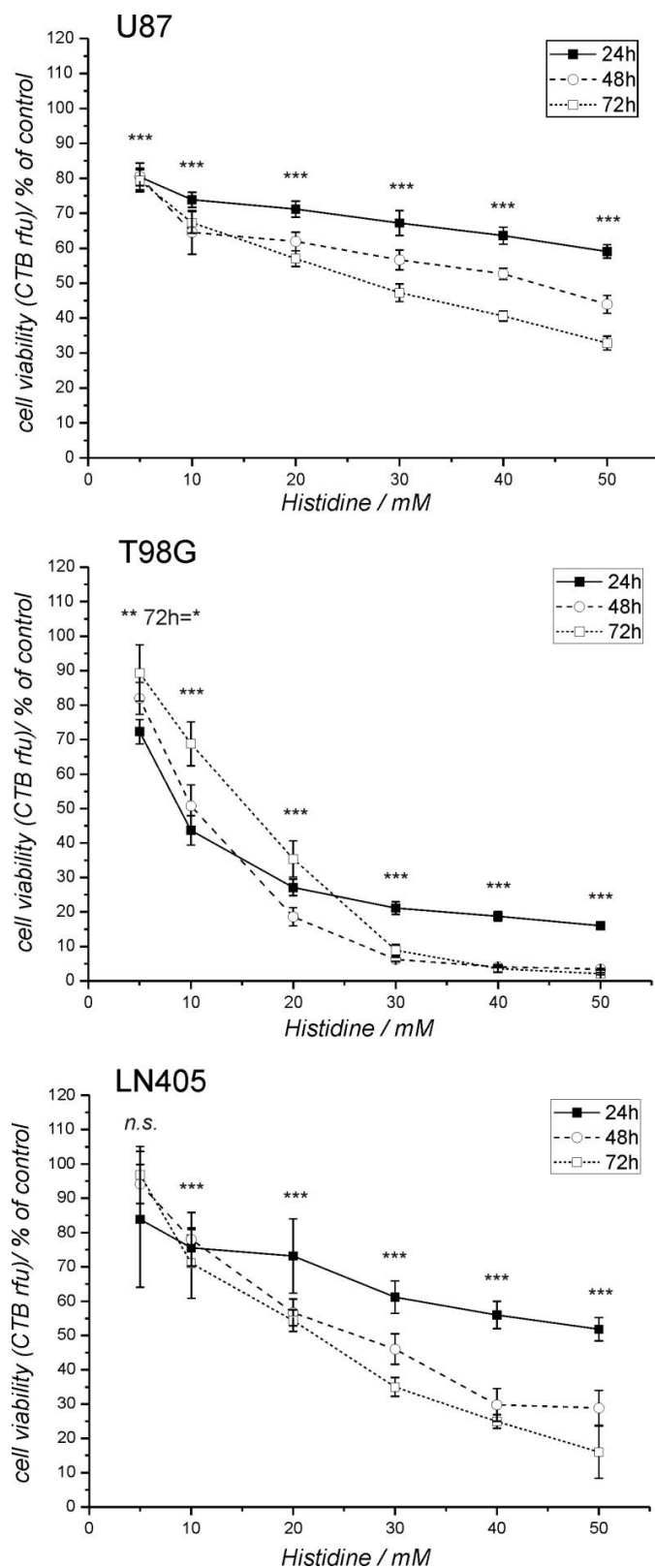
#### **4.1.2 Dehydrogenase activity under the influence of carnosine, L-histidine and $\beta$ -alanine**

Cell viability was further assessed by detecting dehydrogenase activity using the CellTiter-Blue™ assay. Carnosine incubation significantly reduced viability in all three cell lines, as can be seen in Figure 5. This result was significant in all cell lines from 10 mM upwards after 24 hours. The strongest response was detected in T89G, where dehydrogenase activity decreased to 58% ( $\pm$  2%) of the untreated control after 48 hours incubation with 50 mM carnosine. Under the same conditions U87 and especially LN405 showed a weaker response, their dehydrogenase activities being lowered to 67% ( $\pm$  5%) of control in U87 and 71% ( $\pm$  5%) in LN405. A small increase in dehydrogenase activity which appeared in LN405 incubated with 5 mM carnosine after 48 and 72 hours was not significant. After 72 hours incubation with 50 mM carnosine, viability in all cell lines was 60% or less of the viability of the untreated control. Similar to the response seen in ATP production, also a stronger decrease could be detected when dehydrogenase activities in the presence of L-histidine (Figure 6) are compared to the response to carnosine (Figure 5). Starting at 5 mM L-histidine for U87 and T98G and 10 mM for LN405, the dehydrogenase activity measured was significantly lower than that found in the untreated control after 24 hours. After 72 hours incubation with 50 mM L-histidine, the level had fallen to less than 40% of the control in all cell lines. Interestingly, the differences in susceptibility for L-histidine that were seen in ATP production were also reflected in dehydrogenase activities. Here again, U87 and LN405 reacted with a less steep drop in dehydrogenase activity than T98G, in which dehydrogenase activity had decreased to 44% ( $\pm$  4%) of the control at a concentration of 10 mM after 24 hours, while under similar conditions U87 showed 73% ( $\pm$  2%) and LN405 76% ( $\pm$  5%) of the dehydrogenase activity of their respective control.



**Figure 5: Dehydrogenase activity after incubation with carnosine**

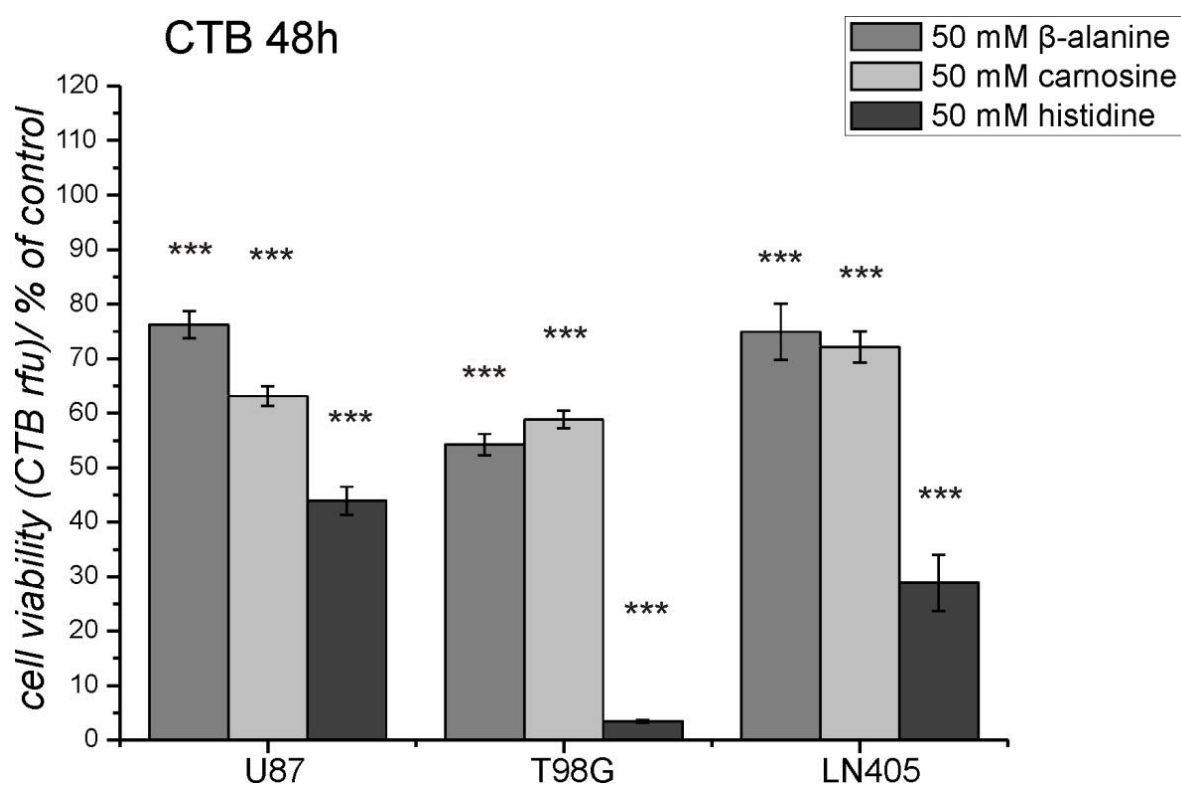
Cells of the lines U87, T98G and LN405 were incubated with 0, 5, 10, 20, 50 and 75 mM carnosine. After 24, 48 and 72 hours dehydrogenase activity was measured by the CellTiter-Blue™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significance is marked by asterisks: \* =  $p < 5 \times 10^{-2}$ ; \*\* =  $p < 5 \times 10^{-3}$ ; \*\*\* =  $p < 5 \times 10^{-4}$ ; n.s. = not significant as calculated by Student's *t*-test.



**Figure 6: Dehydrogenase activity after incubation with L-histidine**

Presented are dehydrogenase activities in cells of the lines U87, T98G and LN405. Cells were incubated with 0, 5, 10, 20, 30, 40 and 50 mM L-histidine. After 24, 48 and 72 hours dehydrogenase activity was determined by the CellTiter-Blue™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significance is marked by asterisks: \* =  $p < 5 \times 10^{-2}$ ; \*\* =  $p < 5 \times 10^{-3}$ ; \*\*\* =  $p < 5 \times 10^{-4}$ ; n.s. = not significant as calculated by *Student's t-test*.

To complete the picture, again carnosine's second component  $\beta$ -alanine had to be included in the experiment. Therefore, cells of the three lines LN405, T98G and U87 were incubated with 0, 20 and 50 mM of  $\beta$ -alanine for 24, 48 and 72 hours. The dehydrogenase activity measurements performed on these cells lead to a different result as seen for ATP production in the presence of  $\beta$ -alanine. In all three cell lines, incubation with 50 mM  $\beta$ -alanine elicited a decrease in dehydrogenase activity compared to the untreated control, the extent of which was similar or slightly stronger compared to 50 mM carnosine for LN405 and T98G. In U87, incubation with carnosine resulted in a more pronounced decrease compared to  $\beta$ -alanine. The reduction achieved by L-histidine again exceeded the one after incubation with the same concentrations of carnosine or  $\beta$ -alanine. In Figure 7, the results for incubation with 50 mM of each substance for a period of 48 hours are shown. Comparable results were obtained with concentrations of 20 mM and incubation times of 24 or 72 hours (data not shown).



**Figure 7: Dehydrogenase activity after incubation with carnosine, L-histidine and  $\beta$ -alanine (48 h)**

The figure shows an overview of dehydrogenase activities measured in cells of the lines U87, T98G and LN405 after 48 hours of incubation with 50 mM of either carnosine, L-histidine or  $\beta$ -alanine. Dehydrogenase activity was detected by the CellTiter-Blue™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significant difference to the respective controls is marked by asterisks: \*\*\*=  $p < 5 \times 10^{-4}$ ; n.s.= not significant as calculated in *Student's t-test*.

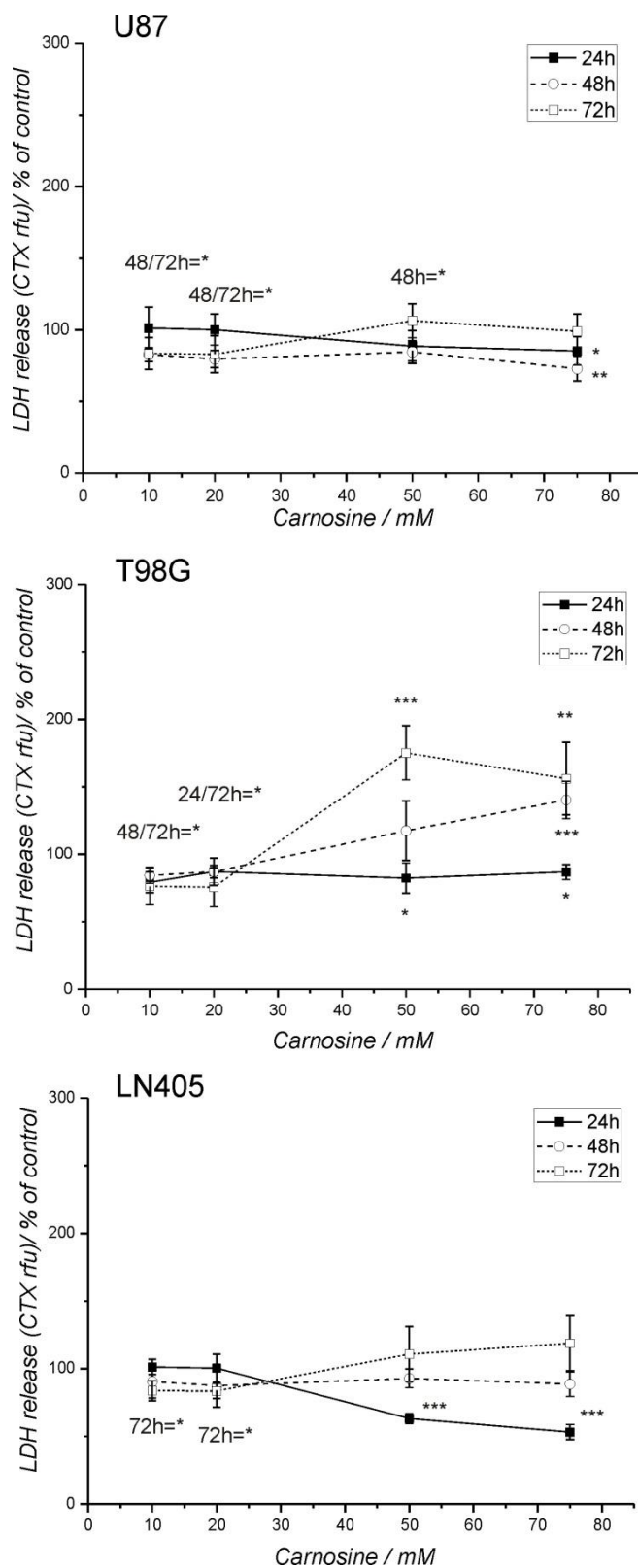
#### **4.1.3 Lactate dehydrogenase activity and necrotic cell death under the influence of carnosine, L-histidine and $\beta$ -alanine**

The measurement of LDH activity in the medium by the CytoTox-ONE™ assay (CTX), which indicates LDH release caused by necrotic cell death, showed no significant elevation in U87 and LN405 after treatment with carnosine. Instead, for both cell lines a significantly reduced activity was detected, which at its lowest point reached 73% ( $\pm$  8%) of the control in U87 incubated with 75 mM carnosine for 48 hours and 53% ( $\pm$  6%) of the control in LN405 incubated with 75 mM for 24 hours (Figure 8). Despite of this fact, no clear tendency towards an increase or decrease of LDH activity related to carnosine incubation could be established. Only in T98G a concentration of 75 mM lead to a significant rise in LDH activity after 48 hours and 72 hours. At 72 hours, this reaction could also be seen in cells incubated with 50 mM, as shown in

Figure 8.

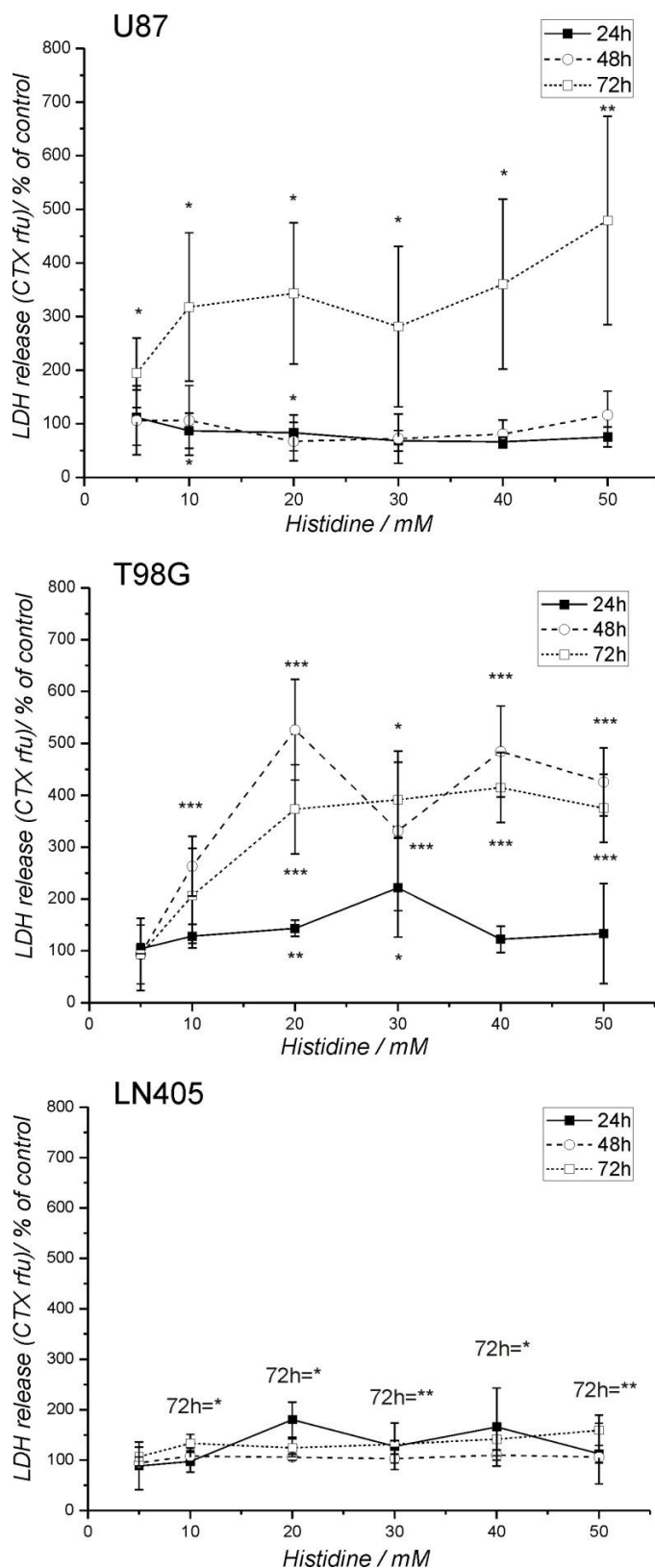
Incubation with L-histidine induced necrotic cell death in all lines, but the intensity and time of onset of this effect varied markedly (Figure 9). T98G appeared to be most susceptible, with LDH activity in the medium rising significantly after 48 hours from 10 mM upwards. At 24 hours, results were only significantly higher for 20 mM and 30 mM. In U87 and LN405 the rise in LDH activity did not appear before 72 hours of incubation. With 159% ( $\pm$  30%) of control in LN405 and 237% ( $\pm$  96%) in U87 incubated with 50 mM L-histidine the increase in activity in these two cell lines was much less drastic than the one seen in T98G, where under the same conditions LDH activity reached 374% ( $\pm$  66%) of the untreated control. In both U87 and LN405 L-histidine caused a reduction of necrosis in the beginning, as exhibited in Figure 9.

It is therefore likely that the negative effect of carnosine on cell viability, which is reflected in lower ATP production and dehydrogenase activity, does not primarily lead to necrotic cell death. L-histidine on the other hand induced necrosis in all three cell lines after a certain time, but only in T98G, which was also most susceptible regarding ATP production and dehydrogenase activity, this effect was present from the beginning of the measurements.



**Figure 8: LDH release after incubation with carnosine**

The figure shows LDH release by cells of the lines U87, T98G and LN405, which had been incubated with 10, 20, 50 and 75 mM of carnosine. After 24, 48 and 72 hours LDH activity in the supernatant was detected by the CytoTox-ONE™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significance as calculated in *Student's t-test* is marked by asterisks:  $\ast = p < 5 \times 10^{-2}$ ;  $\ast\ast = p < 5 \times 10^{-3}$ ;  $\ast\ast\ast = p < 5 \times 10^{-4}$ . All unmarked results were not significantly different from their respective untreated control.

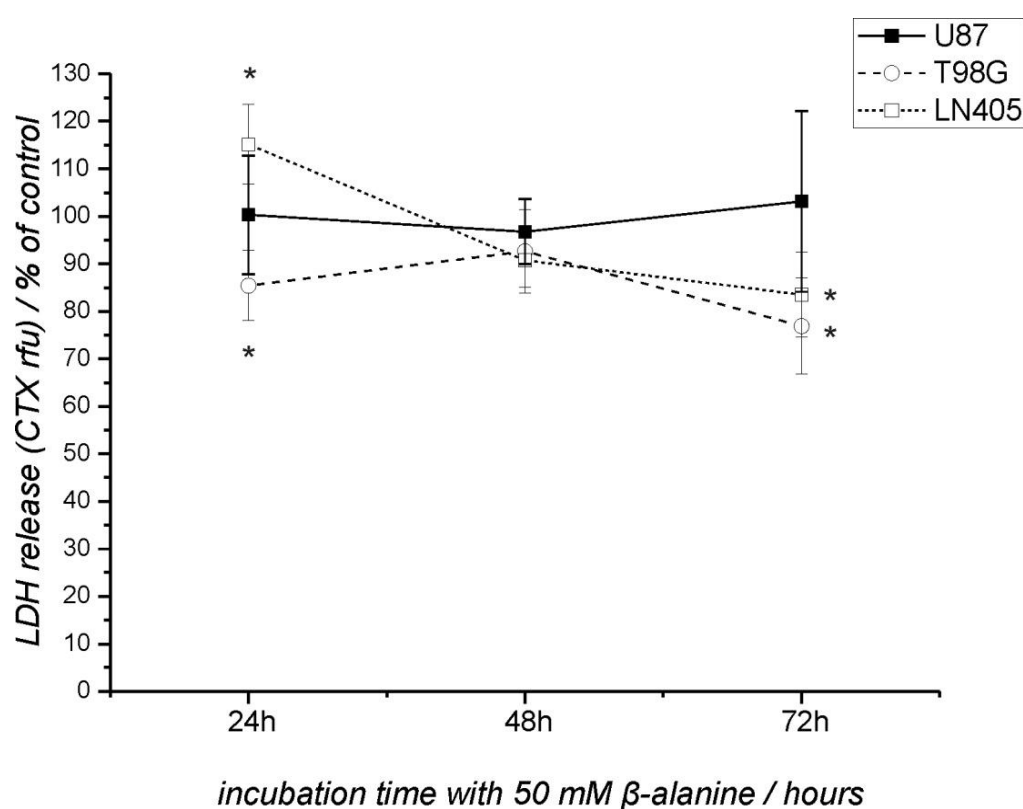


**Figure 9: LDH release after incubation with L-histidine**

Depicted is the LDH release by cells of the lines U87, T98G and LN405, which had been incubated with 5, 10, 20, 30, 40 and 50 mM of L-histidine. After 24, 48 and 72 hours LDH activity in the supernatant was detected by the CytoTox-ONE™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significance as calculated in *Student's t-test* is marked by asterisks: \* =  $p < 5 \times 10^{-2}$ ; \*\* =  $p < 5 \times 10^{-3}$ ; \*\*\* =  $p < 5 \times 10^{-4}$ . All unmarked results were not significantly different from their respective untreated control.



A possible induction of necrosis by  $\beta$ -alanine was also assessed for comparison with the effects of carnosine and histidine. Incubation with 50 mM  $\beta$ -alanine resulted in no significant change in LDH activity in U87. In T98G, a decline to 85% ( $\pm$  7%) and 76% ( $\pm$  10%) of the untreated control could be detected after 24 and 72 hours, but not at 48 hours. LN405 showed an initial rise to 115% ( $\pm$  8%) of the activity measured in the untreated control, but this change was not significant anymore after 48 hours and changed into a decrease to 83% ( $\pm$  9%) of the untreated control after 72 hours. The data is presented in Figure 10. Since in the majority of measurements no increase in LDH activity could be detected, it can be assumed that  $\beta$ -alanine does not elicit necrotic cell death, even in T98G, which reacted with viability changes after  $\beta$ -alanine treatment in the other cell based assays.



**Figure 10: LDH release after incubation with  $\beta$ -alanine**

The figure shows measurement results for LDH release by cells of the lines U87, T98G and LN405, which had been incubated in the presence of 50 mM  $\beta$ -alanine for 24, 48 and 72 hours. LDH activity was measured in the supernatant by the CytoTox-ONE™ assay. Means of six measurements are presented as percentage of the untreated control; error bars indicate standard deviation of the percentage value. Significance calculated in *Student's t-test* is marked by asterisks: \* =  $p < 5 \times 10^{-2}$ . All unmarked results were not significantly different from their respective untreated control.

#### **4.1.4 Concentration dependence of viability decrease under the influence of carnosine and L-histidine**

In the cell viability experiments, carnosine and especially L-histidine had shown to elicit a strong negative effect on ATP production and dehydrogenase activity in human glioblastoma cells. To further characterize this effect, a mathematical function describing the dependence of the cell viability on the concentration of carnosine or L-histidine was sought, which would allow to express the potency of either carnosine or L-histidine by determining the so-called half maximal effective concentration (EC50). The EC50 is the concentration which induces a response halfway between the baseline and the maximum response after the specified exposure time. For this work, a function was considered to be fitting, if the correlation coefficient of the fit was 0.75 or more and if it could be used to describe the measurements of at least two time points for either dehydrogenase activity or ATP production assays for one agent in one cell line. If several types of mathematical fits met these requirements, the one with the highest correlation coefficient ( $R^2$ ) was chosen. For calculating the fits, the functional algorithms implemented in the Origin 8 software were used.

No function fitting the measurements after 24 hours of incubation could be calculated for both L-histidine and carnosine, and it was also not possible to fit the data obtained with carnosine after 48 and 72 hours of incubation. The results for L-histidine obtained after 48 and 72 hours of incubation are presented in the two tables below. In these tables  $R^2$  represents the correlation coefficient for the calculated function. Table 1 shows the fits for the dehydrogenase activity measurements. The decrease of dehydrogenase activity appears to be related to the L-histidine concentration in an exponential way for U87 and in the shape of a sigmoidal curve for T98G and LN405. The same holds true for the decrease in ATP production, the results of which are listed in Table 2.

Apparently, there is a difference in susceptibility between the three cell lines for L-histidine, as judged by the EC 50 values calculated from the functions. Cells of the lines T98G and LN405 appear to react with a decrease starting at lower concentrations of L-histidine compared to U87 cells.

**Table 1: Curve fits for dehydrogenase activity decrease in relation to concentration of L-histidine**

Cell line	Fit type	R <sup>2</sup>	EC 50
48 hours			
U87	exponential	0.968	15.8 mM (t1)
T98G	sigmoidal	0.997	7.7 mM (x0)
LN405	sigmoidal	0.981	9.4 mM (x0)
72 hours			
U87	exponential	0.985	24.0 (t1)
T98G	sigmoidal	0.997	13.1 (x0)
LN405	sigmoidal	0.987	10.7 (x0)

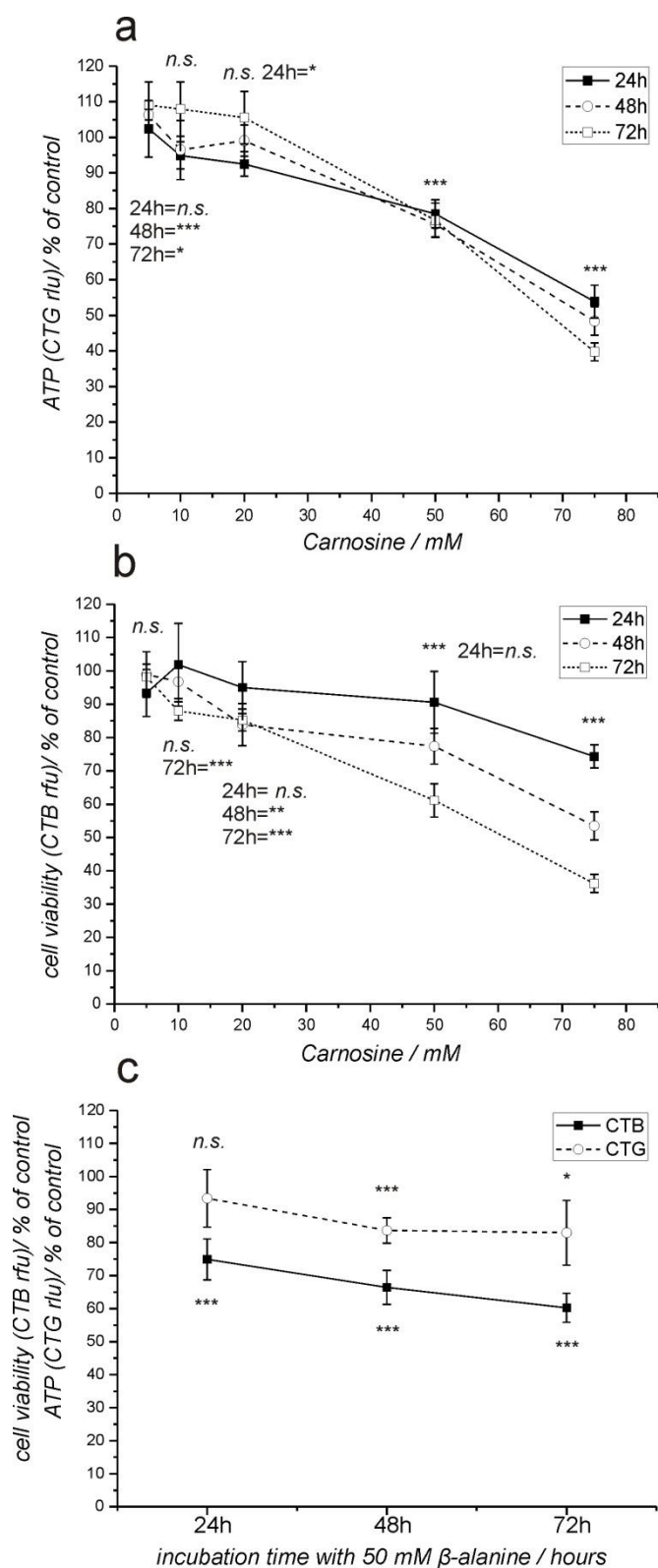
**Table 2: Curve fits of ATP production decrease in relation to L-histidine concentration**

Cell line	Fit type	R <sup>2</sup>	EC 50
48 hours			
U87	exponential	0.770	28.0 mM (t1)
T98G	sigmoidal	0.986	13.2 mM (x0)
LN405	sigmoidal	0.984	16.5 mM (x0)
72 hours			
U87	exponential	0.926	14.7 (t1)
T98G	sigmoidal	0.998	18.2 (x0)
LN405	sigmoidal	0.994	7.1 (x0)

#### **4.1.5 Effect of carnosine and $\beta$ -alanine on viability of HEK 293 cells**

A negative influence of carnosine on the viability of glioblastoma cells is an intriguing finding. However, when considering a potential use of carnosine as an anti-tumour drug, the question needs to be answered whether the effect is specific for tumour cells or whether the viability of non-neoplastic cells is also affected by carnosine in the same way. Therefore, an experiment with non-neoplastic human embryonal kidney cells of the line HEK 293 was performed to assess changes in cell viability after incubation with carnosine and L-histidine. Similar to the glioblastoma cell lines tested before, the cells were seeded on 96-well Greiner microplates, each well containing 5,000 cells, and left to settle in standard medium for 24 hours. After this period, medium was exchanged to establish concentrations of 0, 10, 20, 50 and 75 mM carnosine and 50 mM  $\beta$ -alanine. ATP levels and dehydrogenase activity were determined by the CellTiter-Glo™ and the CellTiter-Blue™ assays after 24, 48 and 72 hours of incubation. As presented in Figure 11, the HEK 293 cells react to incubation with carnosine with a significant drop in ATP levels and dehydrogenase activity. With regard to ATP levels this is significant already for concentrations of 50 mM after 24 hours, where ATP falls to 78% ( $\pm$  4%) of the untreated control. Dehydrogenase activity is significantly decreased to 74% ( $\pm$  3%) at 75 mM after 24 hours and from 20 mM upwards after 48 hours. Incubation with a low concentration of 5 mM carnosine leads to a significant rise in ATP levels to about 106% ( $\pm$  1%) of the untreated control after 48 hours.

Incubation with 50 mM  $\beta$ -alanine also significantly reduces ATP levels and dehydrogenase activity in HEK 293 cells. The intensity of the effect on dehydrogenase activity is similar to that achieved with 50 mM carnosine. On the other hand, ATP levels are not decreased to the same extent as with 50 mM carnosine. The pattern of these effects can be regarded as similar to those seen in T98G, but the intensity is less in the embryonic kidney cells than observed in the glioblastoma cells.



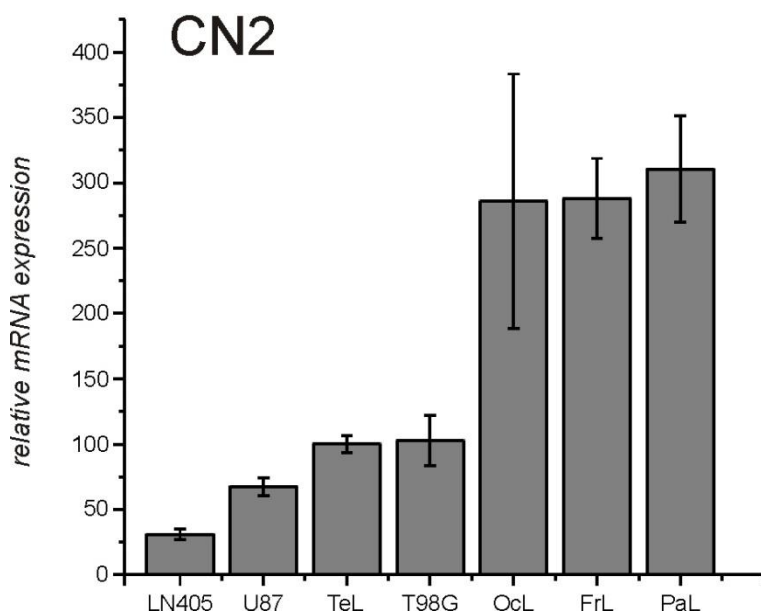
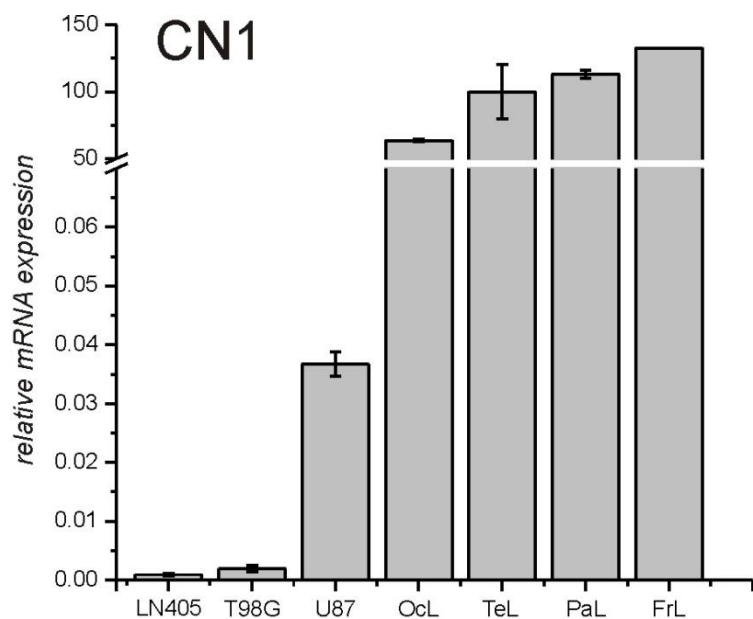
**Figure 11: Viability and ATP production in HEK 293 cells**

Depicted are ATP totals as measured by the CellTiter-Glo™ assay (a) and dehydrogenase activity as determined by the CellTiter-Blue™ assay (b) in cells of the HEK 293 cell line. Cells were incubated for 24, 48 and 72 hours in the presence of 5, 10, 20, 50 and 75 mM carnosine (a, b) or 50 mM  $\beta$ -alanine (c). Means of six measurements are presented as percentage of the untreated control. Error bars indicate standard deviation of the percentage value. Significance as calculated in *Student's t-test* is marked by asterisks: \* =  $p < 5 \times 10^{-2}$ ; \*\* =  $p < 5 \times 10^{-3}$ ; \*\*\* =  $p < 5 \times 10^{-4}$ ; n.s. = not significant.

## 4.2 Carnosinase mRNA expression

Similarities in the effects of carnosine and histidine on cell viability gave rise to the question of whether or not the glioblastoma cells express the enzymes necessary for cleaving L-histidine from carnosine. The two carnosinases CN1 (serum carnosinase) and CN2 (tissue carnosinase) have been described in the literature as being responsible for degrading carnosine in the human body. For the present experiment, CN1 and CN2 mRNA expression was quantified by qRT-PCR using cDNA derived from T98G, LN405 and U87 cells as described above (see 3.3.2). In addition, cDNA from normal brain tissue taken from parietal, frontal, occipital and temporal lobe was analysed. For comparison and normalization, the copy number of TBP mRNA was determined in all samples. To facilitate graphic display, relative expression in the temporal lobe was set to 100% and all other expression levels were depicted in relation to it. The result of this can be seen in Figure 12. Evidently, mRNAs of both types of carnosinase are expressed in all cell lines and cDNA samples tested, suggesting an overall ability to cleave L-histidine from carnosine. CN2 relative mRNA expression is generally higher compared to CN1. Interestingly, all glioblastoma cell lines expressed much lower levels of both CN1 and CN2 mRNA than most normal brain tissue samples. In both LN405 and T98G, mRNA encoding serum carnosinase CN1 was expressed only slightly above the limit of measurement. U87 showed a higher level of expression, which nevertheless was still more than 2000 times below the expression measured in normal brain tissue.

For CN2, expression levels were not as drastically different, with T98G even expressing mRNA on an equal level with temporal lobe tissue. Yet again, a general tendency of higher mRNA expression in normal brain tissue could be observed. Expression levels in occipital, parietal and frontal lobe tissue were approximately 3 times higher than those found in T98G and temporal lobe samples. Of the three glioblastoma cell lines tested, LN405 showed the lowest mRNA expression for CN2.



**Figure 12: Relative CN1 and CN2 mRNA expression**

Relative expression of mRNA encoding CN1 (serum carnosinase) and CN2 (tissue carnosinase) was measured by qRT-PCR, normalized to TBP mRNA expression and depicted as percentage of the expression found in the temporal lobe sample. Tested cDNA included human glioblastoma cell lines LN405, U87 and T98G as well as normal brain tissue from the frontal (FrL), occipital (OcL), parietal (PaL) and temporal lobe (TeL).

### **4.3 PDK4-mRNA expression under the influence of L-histidine**

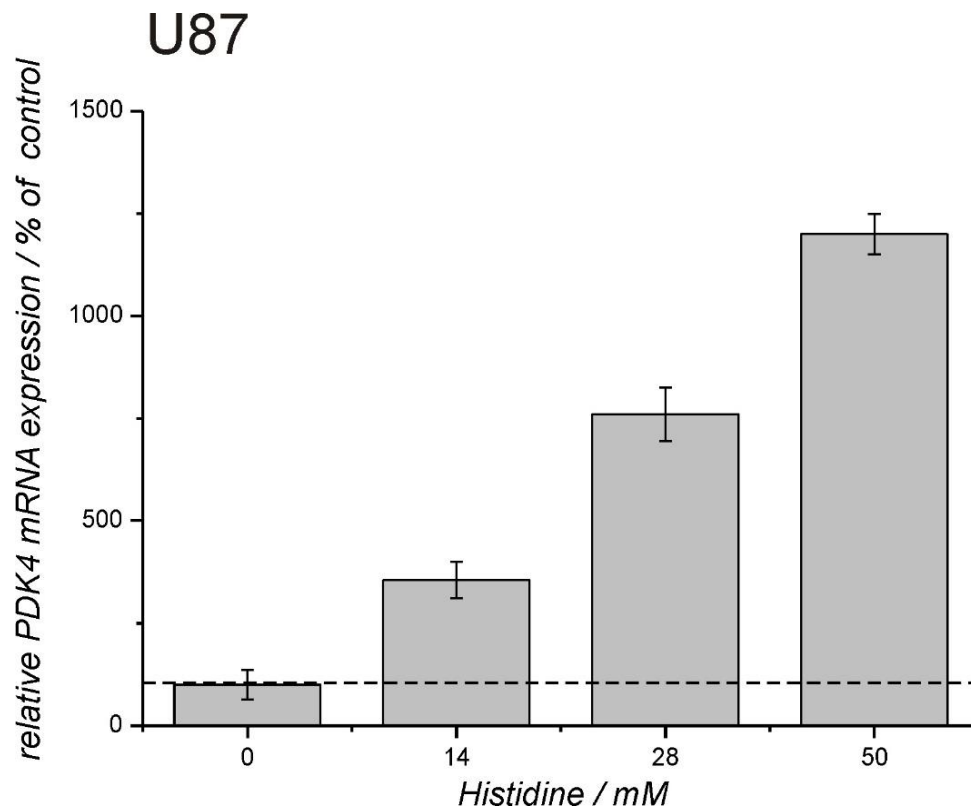
In the cell viability experiments L-histidine was able to affect cell viability and ATP production of glioblastoma cells as seen for carnosine and to an even greater extent. This led to the question of whether L-histidine, like carnosine, can also influence PDK4-mRNA expression in glioblastoma cells. The stimulating effect of carnosine on mRNA expression of PDK4 in human glioblastoma cell lines has been demonstrated before in mRNA-isolation experiments performed by Oppermann *et al.* [8]. In order to investigate possible likewise capacities of L-histidine, similar mRNA-isolation experiments on cells incubated in the presence of L-histidine were performed. For the experiments the cell line U87 was chosen, since this cell line had shown the strongest enhancement of PDK mRNA-expression under the influence of carnosine in the experiments by Oppermann *et al.*

#### **4.3.1 Enhancement of PDK4-mRNA-expression under the influence of L-histidine**

The question of interest in the first mRNA-isolation experiment was whether or not there were detectable changes in PDK4-mRNA expression of glioblastoma cells after incubation with L-histidine. Therefore, cells of the human glioblastoma cell line U87 were incubated with L-histidine for 24 hours. The previously calculated EC<sub>50</sub> of L-histidine (with regard to the CellTiter-Glo™ Assay at 48 and 72 hours of incubation: 14.7 mM to 28.0 mM for U87, adjusted to 14/28 mM for comparability) was used for incubation. In addition, a concentration of 50 mM for U87 was used to allow a comparison with similar concentrations of carnosine from earlier experiments. The cells had been seeded on 10 cm diameter petri dishes at a density of  $1 \times 10^6$  cells per dish in 10 ml standard medium. After being left to settle for 24 hours in an incubator, medium was exchanged to establish the aforementioned conditions. Another 24 hours later, mRNA isolation, reverse transcription and qRT-PCR were performed. An untreated control was included in the experiment and a fourfold measurement was done of all samples. TBP-mRNA was measured in a twofold measurement and used for normalization. Synthesis of cDNA and qRT-PCR was performed twice on each mRNA sample.

Cells treated with L-histidine showed a significant increase in PDK4-mRNA expression, which is presented in Figure 13 as relative copy number of PDK4 in percentage of the untreated control. This effect was as strong as reaching 1199.9% ( $\pm 49\%$ ) percent of the untreated control's relative copy number after incubation with 50 mM L-histidine. A correlation of increase in relative copy numbers to higher concentrations of L-histidine could be seen.



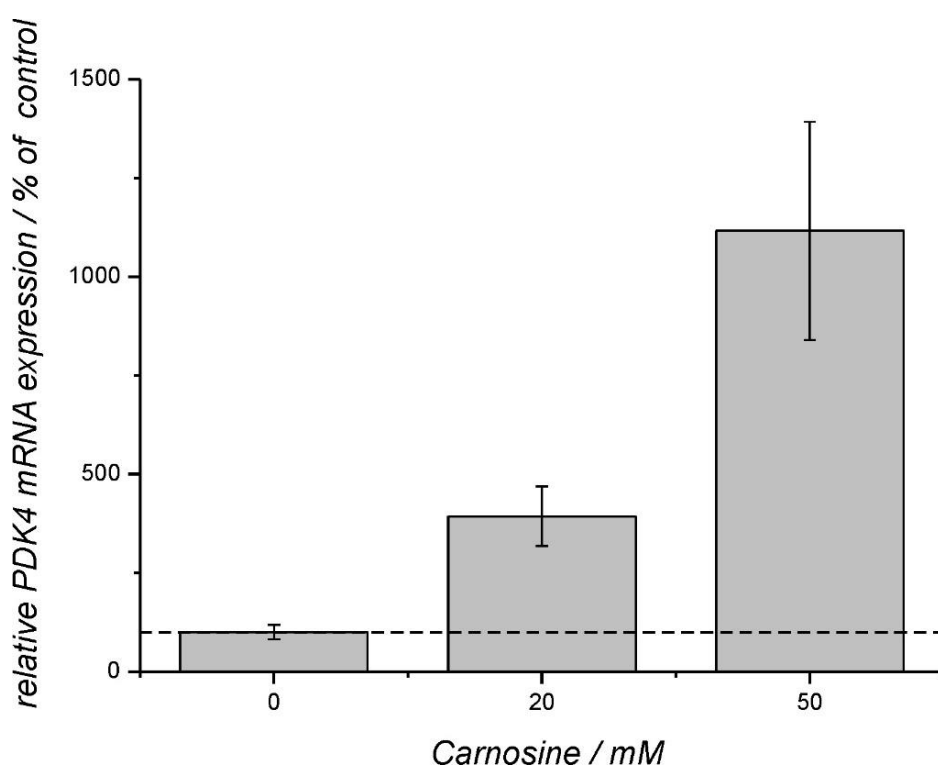


**Figure 13: Relative PDK4-mRNA expression after 24h incubation with L-histidine**

Relative PDK4-mRNA expression was determined by qRT-PCR in cells of the U87 cell line after 24 hours of incubation with L-histidine. Concentrations of 0, 14, 28 and 50 mM L-histidine were employed for incubation. Copy numbers of PDK4-mRNA were normalized to copy numbers of TBP mRNA. Values are given as percentage of the untreated control; error bars represent standard deviation of the percentage value. A dashed line indicates the level of the untreated control to facilitate comparison. Significance for all measurements was at  $p < 0.05$  in *Student's t-test*.

For comparison, the development of PDK4-mRNA expression in U87 cells after 24 hours of incubation with 20 mM and 50 mM carnosine is presented in Figure 14. After being seeded at a density of  $1 \times 10^6$  cells per dish on 10 cm diameter petri dishes in 10 ml standard medium and settling for 24 hours, these cells had received standard medium containing 0, 20 and 50 mM of carnosine. Isolation of mRNA was undertaken another 24 hours later, followed by reverse transcription. Then, qRT-PCR was performed with a fourfold measurement of each sample for PDK4-mRNA and a twofold measurement for TBP-mRNA, which was used for normalization. Synthesis of cDNA and qRT-PCR was again performed twice on each mRNA sample.

Here as well, a significant rise of relative copy numbers was measured that also appears to gain in intensity in 50 mM compared to 20 mM. Relative expression of PDK4-mRNA reached 1116% ( $\pm 276\%$ ) of the untreated control after incubation with 50 mM carnosine and 394% ( $\pm 75\%$ ) with 20 mM carnosine.

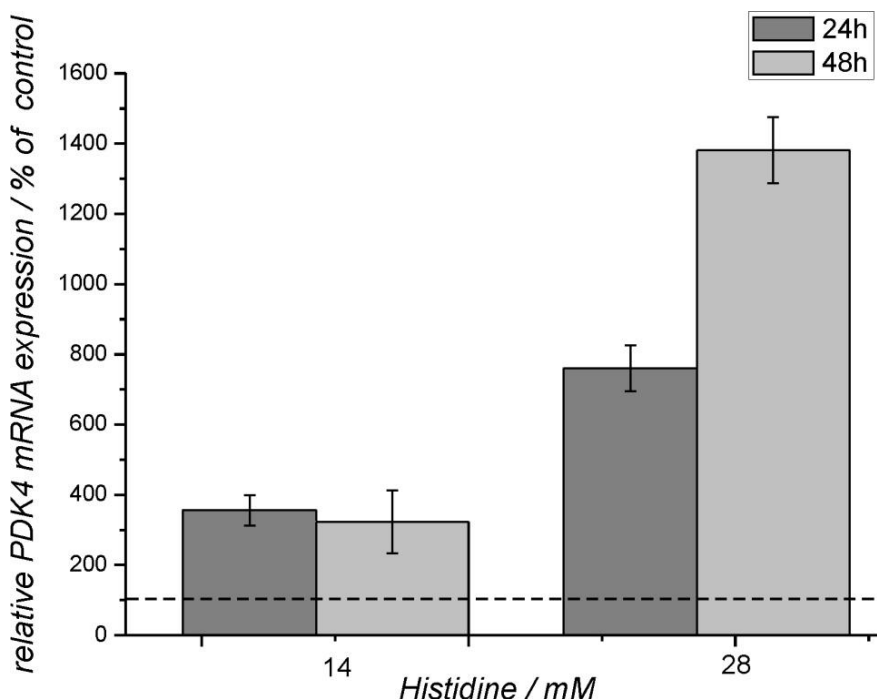


**Figure 14: Relative PDK4-mRNA expression in U87 cells after 24h incubation with carnosine**

Relative PDK4-mRNA expression was measured by qRT-PCR in U87 cells after 24 hours of incubation with 0, 20 and 50 mM carnosine. Copy numbers of PDK4-mRNA were normalized to copy numbers of TBP mRNA. Values are given as percentage of the untreated control; error bars represent standard deviation of the percentage value. A dashed line indicates the level of the untreated control to facilitate comparison. Significance for all measurements was at  $p < 0.05$  in *Student's t-test*.

#### 4.3.2 Development of L-histidine-mediated PDK4-mRNA increase over time

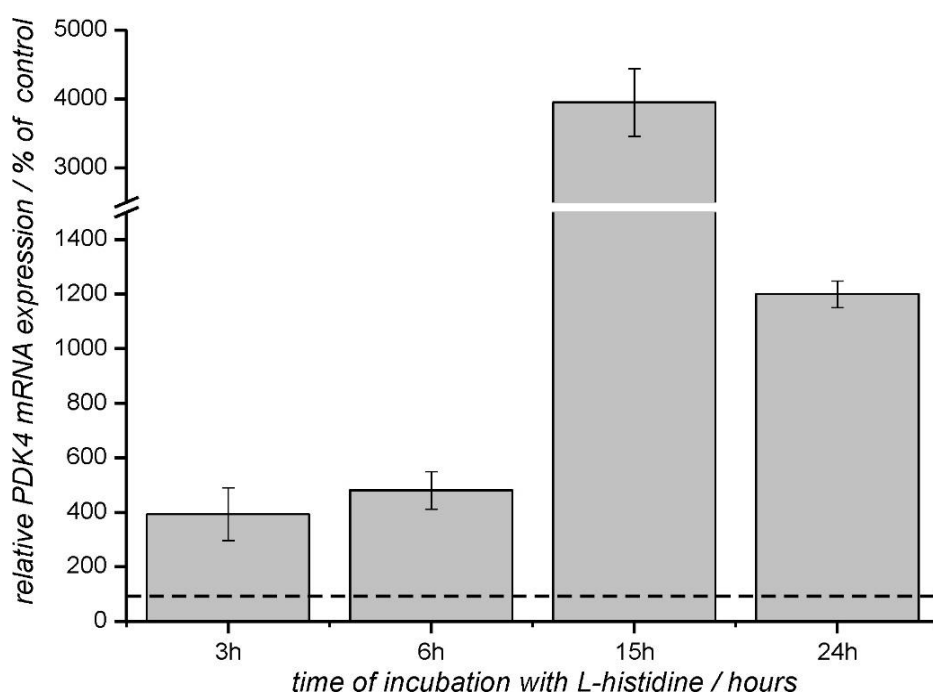
In order to analyse whether there are differences between the effect of carnosine and L-histidine on PDK4 expression it was tested whether both compounds influence expression of PDK4 with an equal time-kinetic. As shown before by Oppermann *et al.*, PDK4-mRNA expression in U87 human glioblastoma cells was enhanced by incubation with 50 mM carnosine after 24 and also after 48 hours, but the enhancement after 48 hours was less intense [8]. It was therefore investigated whether the same could be seen in U87 cell incubated with L-histidine. As in the prior experiments, cells had been seeded in 10 cm petri dishes, each dish containing  $1 \times 10^6$  cells in 10 ml standard medium. All cells had been pre-incubated with standard medium for 24 hours before establishing concentrations of 0, 14 and 28 mM L-histidine. In this experiment again significantly elevated relative copy numbers compared to the untreated control were detected. As Figure 15 shows, the increase in relative copy numbers after 48 hours was not different from 24 hours in the presence of 14 mM L-histidine, in which relative copy numbers at both points of time reached over 300% of the untreated control. In the presence of 28 mM L-histidine the gain was generally stronger with an obvious difference in the level of expression between 24 and 48 hours. Here, relative PDK4-mRNA copy numbers amounted to 760% ( $\pm 65\%$ ) of the untreated control after 24 hours and 1380% ( $\pm 94\%$ ) after 48 hours.



**Figure 15: PDK4-mRNA expression in U87 cells after 24h and 48h incubation with L-histidine**

The figure shows the relative expression of PDK4-mRNA in cells of the line U87 incubated for 24 and 48 hours in medium containing L-histidine (14 and 28 mM) compared to its relative expression in cells cultivated in standard medium (0.2 mM L-histidine, set to 100%). To calculate the relative expression, PDK4 and TBP mRNA copy numbers were determined by qRT-PCR using standard curves and the relative expression determined by comparing PDK4 copy number to the copy number of TBP. Error bars represent standard deviation of the percentage value. The dashed line indicates the level of the untreated control to facilitate comparison. Significance for all measurements was at  $p < 0.05$  in Student's *t*-test.

Next, it was investigated whether changes in PDK4-mRNA-expression under the influence of L-histidine could already be detected after incubation times of less than 24 hours. By understanding this, further insight could be gained on whether short-term or long-term changes in cellular metabolism and gene expression were more likely to be involved in mediating L-histidine's effect. In this experiment, cells of the line U87 were incubated in the presence of L-histidine for 3, 6, 15 and 24 hours. For this experiment, it was decided to use a concentration of 50 mM L-histidine for short-term incubation in order to raise the likelihood of a measurable effect. Again, at each point of time, mRNA from an untreated control was isolated for reference. In Figure 16, which presents the result of this experiment as relative PDK4-mRNA expression in percentage of the untreated control, it can be seen that expression rises significantly already 3 hours after adding L-histidine. At this point, it reaches 280% ( $\pm 69\%$ ) of the control. The effect appears to be strongest at 15 hours and declines towards 24 hours.



**Figure 16: PDK4-mRNA expression in U87 cells after short-term incubation with L-histidine**

The figure presents relative PDK4-mRNA expression in cells of the line U87, which had been incubated in the presence or absence of 50 mM L-histidine for 3, 6, 15 and 24 hours. Relative copy numbers are expressed as percentage of the relative copy numbers in the untreated control, which was set to 100%. The untreated control received medium containing 0.2 mM L-histidine. To calculate relative expression, copy numbers of PDK4-mRNA and TBP mRNA were determined by qRT-PCR using standard curves and the ration of PDK4-mRNA copy numbers to copy numbers of TBP mRNA was calculated. Error bars represent standard deviation of the percentage value. The dashed line indicates the level of the untreated control to facilitate comparison. Significance for all measurements was at  $p < 0.05$  in *Student's t-test*.

Seen in synopsis with the 48 hour experiment, this allows the conclusion that L-histidine induces an increase in expression of PDK4-mRNA in U87 cells, which gains intensity over time, the exact peak point of time being variable among different concentrations of the agent. A biphasic pattern with peaks before and after 24 hours is also possible. A decrease of expression enhancement after incubation for 48 hours, as reported for 50 mM carnosine by Opperman *et al.*, could not be detected for L-histidine.

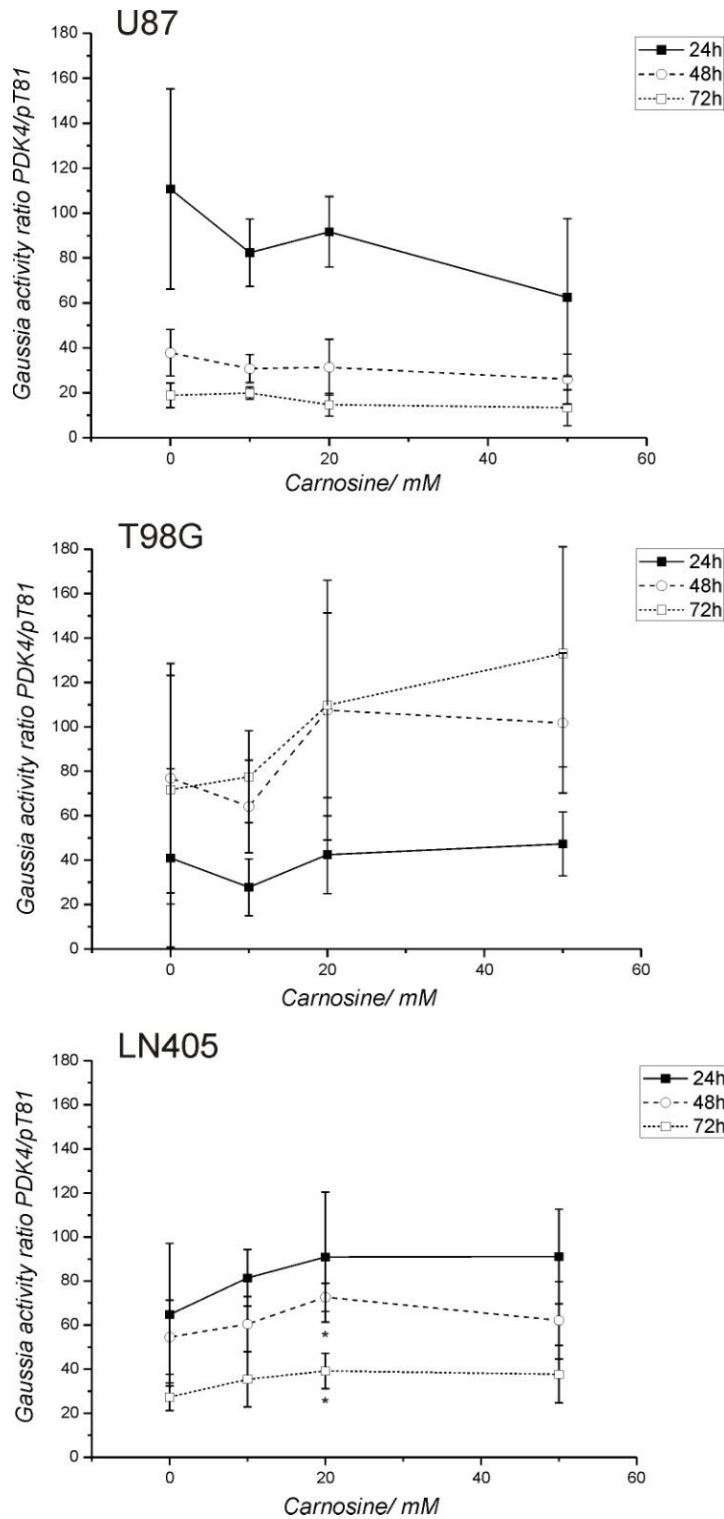
## 4.4 Reporter gene assays

In order to analyse whether the effects of L-histidine and carnosine on PDK4 expression are mediated by cis-regulatory elements within the PDK4 promoter region, a reporter gene with DNA sequences from the PDK4 gene controlling the expression of the *Gaussia* luciferase gene was constructed. The reporter gene contained a sequence from the human PDK4 gene encompassing a region from +3968 bp upstream to +319 bp downstream from the transcription starting point. The downstream part ends with start codon (ATG) which in this construct was the start codon of the luciferase of *Gaussia princeps*, whose expression can be quantified by luminescence measurement. Since the luciferase gene was controlled by the human PDK4 promoter in the constructed plasmid, factors enhancing expression at the promoter would also cause increased expression of the luciferase. In this way, enhancement mediated by elements located within the promoter can be quantified by luminescence measurement. The complete reporter gene plasmid was named *(-3968/+319)\_PDK4\_GaulIII* (for further details on the construction process see 3.4). For comparison, another plasmid termed *pT81\_GaulIII* was used in the experiments. It contained only the *pT81* vector and the *Gaussia princeps* luciferase gene and could therefore help to separate general effects on reporter gene expression from effects related specifically to the human PDK4 promoter and 5' UTR region.

In the experiment, cells from the lines U87, LN405 and T98G were transfected with either the *(-3968/+319)\_PDK4\_GaulIII* plasmid or the *pT81\_GaulIII* plasmid using the cationic polymer transfection agent Turbofect as described above (see 3.3.1). After 100 minutes of exposure to the transfection reagent/DNA mixture, medium was exchanged to contain 0, 10, 20 or 50 mM carnosine. 24, 48 and 72 hours after the medium exchange, *Gaussia* luciferase activity in 10 µl of the supernatant from each well was measured and medium was exchanged maintaining the same conditions. At 72 hours, a CellTiter-Glo™ viability assay was performed as well to determine ATP production in the cells.

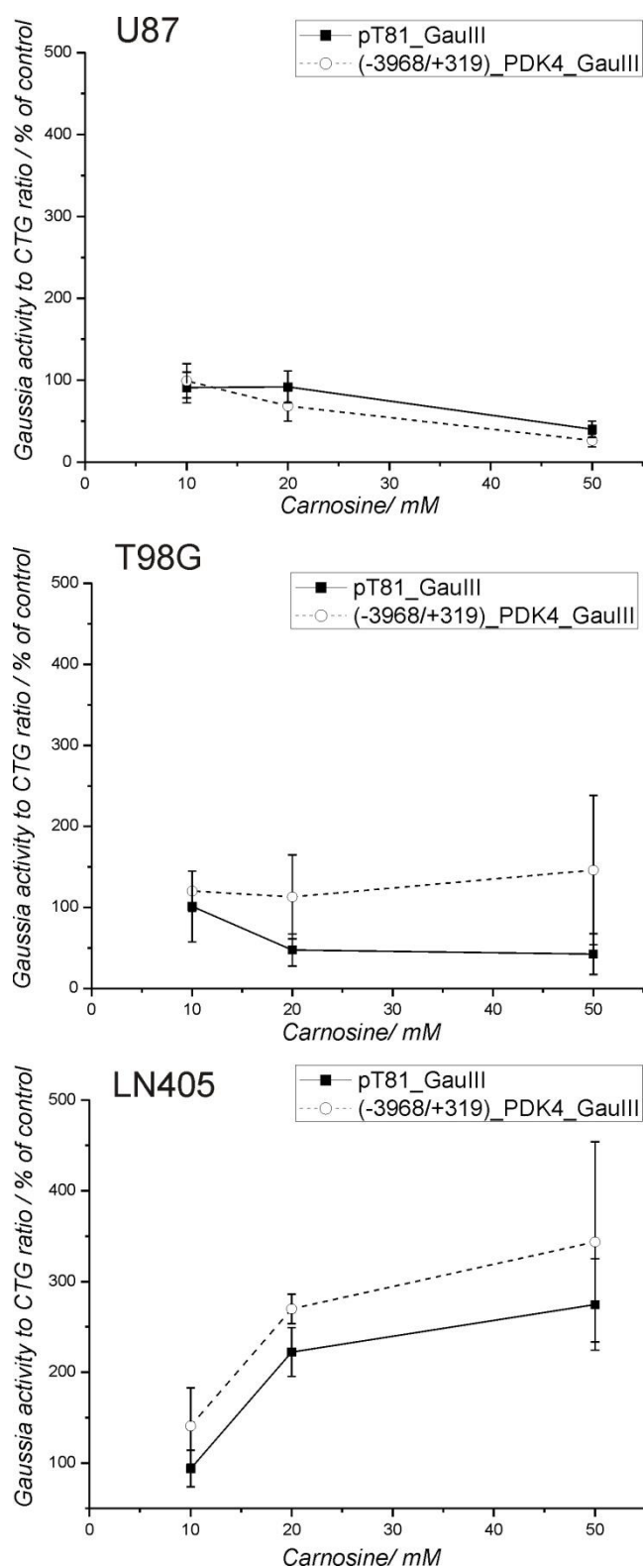
The ratio of *Gaussia* activity of cells transfected with the PDK4 plasmid to that of cells transfected with *pT81\_GaulIII* was calculated in order to distinguish increases or decreases in the PDK4 reporter gene activity from general changes in activity affecting all cells. As outlined in Figure 17, the ratio of PDK4 plasmid to *pT81\_GaulIII* plasmid *Gaussia* activity showed a diverse picture of gain and loss for different concentrations of carnosine, but none of these changes reached a significant level.

The additional measurement of ATP production allowed a comparison of cell viability and *Gaussia* activity, which showed that *Gaussia* activity of the two plasmids is mainly parallel to differences in cell viability (Figure 18). This leads to the conclusion that apart from a general effect on cell viability, carnosine did not differentially influence the expression of the control vector and the PDK4 reporter gene.



**Figure 17: Reporter gene expression after incubation with carnosine**

Cells of the lines U87, T98G and LN405 were transfected with either the *(-3968/+319)\_PDK4\_GauIII* plasmid or with *pT81\_GauIII* plasmid, which did not contain the *(-3968/+319)\_PDK4* insert. The transfected cells were then incubated for 24, 48 and 72 hours in the presence of 0, 10, 20 and 50 mM carnosine. After each interval, *Gaussia princeps* luciferase activity was determined by luminescence measurement to quantify expression of either plasmid. Then for each cell line and each condition, ratios of activities measured in either plasmid group were calculated. Ratios are given as means of six measurements; error bars indicate standard deviation of ratios. Significant changes are marked by asterisks:  $\ast = p < 5 \times 10^{-2}$  in Student's *t*-test.



**Figure 18: Reporter gene expression in relation to cell viability after incubation with carnosine**

Ratio of reporter gene activity to ATP amounts in U87, T98G and LN405 cells transfected with the (-3968/+319)\_PDK4\_GaulIII plasmid and cells transfected with the pT81\_GaulIII plasmid. Cells had been incubated with 0, 10, 20 and 50 mM carnosine for 72 hours. Ratios are expressed as percentage of the untreated control, which is set to 100%. Error bars indicate percentage error.

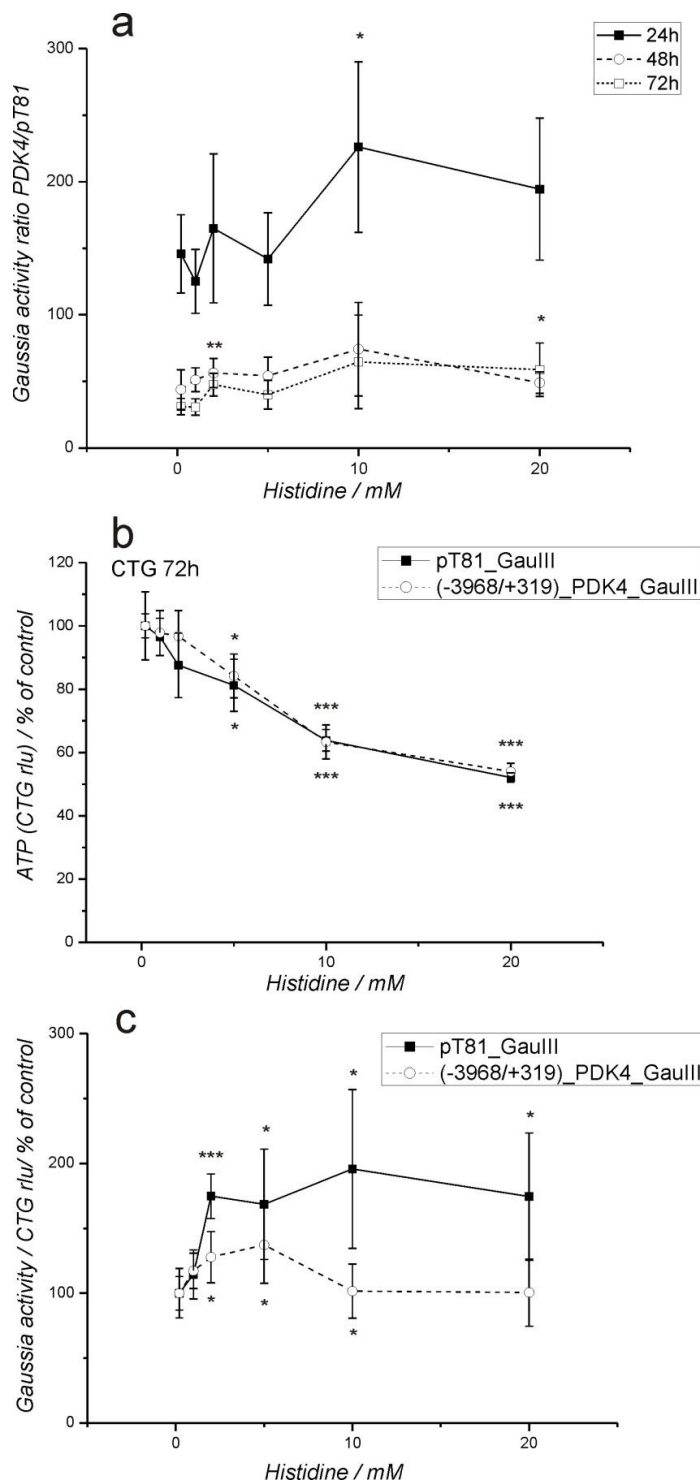


The experiment was repeated with 50 mM carnosine for intervals of up to 72 hours with U87 and T98G cells. An overview of the number of experiments and their results can be found in Table 3. Apparently, in the majority of experiments no significant change in expression of the PDK4 reporter gene could be measured and none of the experiments showed a significant increase after carnosine treatment, as would be expected from the enhanced expression of the endogenous PDK4 gene under the influence of carnosine.

**Table 3: Reporter gene assays with 50 mM carnosine in U87 and T98G**

Cell line	Incubation time	Number of experiments	Significant Increase	Significant Decrease
U87	24h	5	0	2
	48h	3	0	0
	72h	3	0	1
T98G	24h	3	0	1
	48h	2	0	0
	72h	1	0	0

In a second experiment, 0, 1, 2, 5, 10 or 20 mM L-histidine was applied to cells of the line U87, which were transfected and treated in the same way as described above for carnosine. It has to be mentioned that the standard medium used in all experiments already contained 0.2 mM L-histidine as stated by the manufacturer. Again, *Gaussia* activity in 10 µl of medium was measured after 24, 48 and 72 hours and the amount of ATP produced by the cells was determined after 72 hours. The ratio of luciferase activity in cells transfected with the (-3968/+319)*\_PDK4\_GauIII* plasmid to those transfected with *pT81\_GauIII* was also calculated. The results, as shown in Figure 19, once more did not point towards a specific effect on the expression of the PDK4 reporter gene. In most concentrations of L-histidine, no significant change in the calculated ratios could be detected. Although there were three measurements showing a significant increase, this does not appear to stand in any clear relation to L-histidine concentrations. The diminishing effect on ATP levels was the same in cells transfected with either plasmid. Regarding the ratio of reporter gene *Gaussia* activity to ATP levels measured in CellTiter-Glo™, the cells expressing (-3968/+319)*\_PDK4\_GauIII* reacted with a slight increase similar to those transfected with *pT81\_GauIII*.



**Figure 19: Reporter gene activity after incubation with L-histidine**

Cells of the line U87 were either transfected with the (-3968/+319)\_PDK4\_GauIII plasmid or the pT81\_GauIII plasmid. Gaussia activity was measured after 24, 48 and 72 hours of incubation in the presence of 0, 5, 10 or 20 mM L-histidine and ratios for activities from both plasmids were calculated (a). The amount of ATP in the cells was determined by CellTiter-Glo™ after 72 hours (b). Ratios of reporter gene activity to ATP amount after 72 hours were calculated for cells with either plasmid (c). Gaussia activity ratios (a) are given as means of six measurements; error bars indicate standard deviation of ratios. ATP totals (b) and Gaussia activity/ATP ratio (c) are expressed as percentage of the untreated control. Error bars indicate percentage error. Significance as in Student's *t*-test is marked by asterisks: \* =  $p < 5 \times 10^{-2}$ ; \*\* =  $p < 5 \times 10^{-3}$ ; \*\*\* =  $p < 5 \times 10^{-4}$ .

To rule out the possibility that the transfection process itself might hinder the effect of carnosine on endogenous PDK4-mRNA expression, mRNA isolation was performed in (-3968/+319)\_PDK4\_Gaull plasmid transfected and untransfected cells incubated with 0 and 50 mM carnosine for 24 hours. In this experiment, no significant influence of transfection with regard to the enhancement of PDK4-mRNA expression under the influence of carnosine was observed nor was any difference in the overall expression of PDK4-mRNA detected between transfected and untransfected cells (data not shown). It can thus be concluded that carnosine and L-histidine both stimulate endogenous PDK4-mRNA expression. However, the reporter gene experiments did not reveal a contribution of the PDK4 promoter to the effect.

## 5 Discussion

### 5.1 Conclusions

The negative effect of carnosine on viability and proliferation of tumour cells has been shown by different groups of researchers in the past, both *in vitro* and *in vivo*. Following the first description of carnosine-related tumour growth inhibition in a Sarcoma-180 mouse model by Nagai and Suda in 1986 [1], similar effects could also be found in mouse models with NIH3T3-Her2/neu human fibroblasts and human colon cancer cells [2, 83]. *In vitro* experiments with analogous results were conducted for cell lines of pheochromocytoma [5], KRAS-mutated colon cancer cells [6], human carcinoma cells of lung, cervix, bladder and prostate origin and human osteogenic sarcoma [4] among others, and also in cells isolated from patient samples of human glioblastoma [3].

A connection of growth inhibition and lower ATP levels after carnosine treatment was demonstrated both by Renner *et al.* and Iovine *et al.* [7, 3, 6], the former using the human glioblastoma cell line T98G among others. In the experiments presented here, a decrease of ATP levels could also be observed in human glioblastoma cells of the lines U87, LN405 and T98G after treatment with L-histidine. Furthermore, in comparison to cells incubated with carnosine the decline was more marked after L-histidine incubation and appeared at lower concentrations in all three cell lines. Cell viability as measured by dehydrogenase activity was reduced in the same manner both by carnosine and L-histidine, with L-histidine again eliciting a stronger reaction. A pattern of susceptibility could be detected with T98G showing the sharpest drop in ATP production and cell viability after L-histidine treatment and likewise after carnosine treatment, although the difference between the three cell lines was not as marked for carnosine. In addition to that, only T98G reacted to  $\beta$ -alanine treatment in the same way as to carnosine, although the effect of L-histidine was much stronger in these cells at a comparable molarity. U87 and LN405 cells incubated with  $\beta$ -alanine showed a reduction of dehydrogenase activity, but not of ATP production.

It can thus be concluded that L-histidine decreases ATP production and viability in human glioblastoma cells as reported previously for carnosine, and to a stronger effect. This stands in contrast to findings in yeast reported by Cartwright *et al.*, who detected a loss of viability after incubating yeast cells grown in 2% glucose with 10 mM carnosine, but could not reproduce such a loss in cells incubated with 10 mM L-histidine [85]. As for neoplastic cells or tissues of human origin, Holliday and McFarland reported a toxic effect on HeLa-cells both for carnosine and histidine and also for anserine, but not for  $\beta$ -alanine [4].

Necrotic cell death, which can be quantified by measuring LDH activity in the supernatant of cultivated cells, was not induced by carnosine except in concentrations of more than 50 mM. L-histidine on the other hand significantly raised LDH activity after 72 hours in all cell lines and even earlier in T98G.

Cytotoxicity of histidine has been demonstrated before in combination with hydrogen peroxide and several DNA strand breaking agents starting at much lower concentrations as the ones used in our experiments [92, 93], but contrary findings have also been published [94].

The rise in the abundance of PDK4-mRNA in human glioblastoma cells of the U87 line after incubation with carnosine could be shown in the experiments presented in this thesis. This finding is in accordance with experiments by H. Oppermann, who detected PDK4-mRNA increase in U87, T98G and LN405 cells incubated with carnosine [8]. Furthermore, it was possible to demonstrate here that incubation with L-histidine also leads to a rise in PDK4-mRNA copy number in U87 cells. The intensity of this increase was positively correlated to histidine molarity. The effect induced by L-histidine was stronger than that caused by similar molarities of carnosine, although not as evident as seen in the cell based assays. These findings are in accordance with experiments of 20 mM and 50 mM L-histidine and 50 mM carnosine in U87, T98G and LN405 cells after 24 hours of incubation, which were performed by H. Oppermann. In these experiments LN405 reacted with a significant, yet less pronounced rise than U87 and T98G. On the other hand,  $\beta$ -alanine caused no significant change in PDK4-mRNA in U87 cells [8]. An influence of L-histidine on mRNA abundance has previously been described by Son *et al.* for Caco-2 and HT-29 cells, in which L-histidine inhibited the up-regulation of IL-8 expression after treatment with TNF- $\alpha$  or hydrogen peroxide [94]. The same group could report later that carnosine had no effect on IL-8 mRNA abundance in the Caco-2 cell line, unlike L-histidine, anserine and other histidine containing dipeptides tested. Measurements of intracellular histidine in these cells revealed that it was not cleaved from carnosine by the Caco-2 cells [95]. In light of these findings, it is likely that carnosine's effect on transcription is related to L-histidine being cleaved from the dipeptide.

This aspect was also of interest for this study, since there appeared to be differences between the three tested glioblastoma cell lines in their susceptibility to carnosine and L-histidine. This could possibly be explained by differences in expression of transporters carrying carnosine and L-histidine into the cells or of the two carnosinase enzymes CN1 and CN2. The latter are responsible for releasing L-histidine from carnosine. In measurements of mRNA encoding for CN1 and CN2 in the glioblastoma cell lines and samples of normal brain tissue, it could be shown that all three glioblastoma cell lines expressed CN1 and CN2 mRNA. Therefore, they can be considered as being able to cleave L-histidine from carnosine, although this assumption would need to be verified by measurements of L-histidine in the cells and the medium after incubation with carnosine. As a second finding, it was noted that the glioblastoma cells expressed CN1 and CN2 mRNA at a drastically lower level compared to normal tissue. In LN405 and T98G the expression levels of CN1 mRNA were only slightly above the limits of measurement. It is therefore unlikely that CN1 is the key enzyme mediating the effect of carnosine on glioblastoma cells. Interestingly, lowered plasma levels of serum carnosinase in patients with glioblastoma have already been reported [62]. For the cytosolic carnosinase CN2, expression levels

were generally higher. But here again the glioblastoma cell lines expressed this mRNA to a lesser extent compared to normal brain tissue. Expression patterns of mRNA for CN2 could be linked to differences in results of viability and PDK4-mRNA measurements after carnosine treatment. T98G cells expressed the highest level of all three glioblastoma cell lines, followed by U87 and at the least LN405. These differences might offer an explanation for the stronger reaction of this cell line to carnosine treatment and also the only mild influence of carnosine on mRNA expression in LN405. It can be assumed that carnosine acts as a vehicle for L-histidine to be transported into the cells and released there after cleavage by CN2. A role of carnosine as a reservoir for L-histidine was already proposed regarding histamine synthesis [46]. On the other hand, neither the differences in reaction to L-histidine between the cell lines, nor the diversity of responses to carnosine treatment can be explained by this theory alone. Therefore, carnosine is likely to influence mRNA expression and cell viability in other ways than only via releasing L-histidine as a final effector.

Seeing that carnosine and L-histidine elicit a loss in tumour cell viability and also an increase in PDK4-mRNA, the question of a relation between these two observations has to be discussed. A connection of PDC and PDK to the carnosine-induced impairment of viability in neoplastic cells is suggested by the findings of Holliday and McFarland, who demonstrated that pyruvate inhibits carnosine's effect on cell viability [4]. Pyruvate is known to inhibit PDK4 activity [97]. Up-regulated PDK4-mRNA has also been shown to decrease the viability of tumour cells, and PDK4 expression was shown to be generally lower in tumours when compared to their tissues of origin [116]. The finding of decreased ATP levels in neoplastic cells after carnosine treatment [7, 3] is in itself a hint towards an interaction of carnosine with enzymes of glycolysis. Transformed and neoplastic cells appear to be especially susceptible to carnosine, as indicated in experiments with co-cultures of transformed and non-transformed cells, in which carnosine selectively eliminated the transformed cells [4]. Neoplastic cells depend on glycolysis for ATP production [7]. Additionally, a lessening in the availability of acetyl-CoA, which would be the result of up-regulated PDK4, might also affect the growth of the cells since acetyl-CoA is needed for the synthesis of membrane building lipids.

Interestingly, pyruvate does not inhibit the decrease of viability caused by L-histidine [4]. However, in the experiments presented in this thesis a significant influence of L-histidine on PDK4-mRNA was observed. Thus, it may be assumed that the mechanisms by which carnosine and L-histidine influence cell viability and ATP levels are different, but share some common features.

Since a strong enhancement of PDK4 transcription after carnosine and L-histidine incubation could be shown in the mRNA experiments, effects of the two agents on a human PDK4 5'UTR-reporter gene were investigated. The cell lines tested included LN405, U87 and T98G. No significant increase or decrease of reporter gene expression was detected after treatment with either carnosine or L-histidine. Apart from the possibility that the cis-elements responsible for the effect mediated by carnosine lie

outside the sequences covered by the reporter gene, this may also suggest an influence of carnosine on epigenetic mechanisms that are not mirrored by reporter gene experiments or an influence on mRNA stability. At least the experiments performed by Son *et al.* point at the possibility that carnosine may alter expression of IL-8 at a post-transcriptional level [95]. Furthermore, transfected reporter genes differ from endogenous genes in their chromatin structure [131]. This might explain differences in the impact of factors regulating transcription by changing chromatin packaging, for example by histone acetylation or de-acetylation.

The finding that carnosine and L-histidine influence tumour cell viability and expression of PDK4-mRNA in human glioblastoma cell lines is interesting with regard to cancer therapy. However, it needs to be assessed whether this effect is limited to tumour cells as suggested for carnosine by Holliday and McFarland [4]. Here it was shown that the non-neoplastic human embryonic kidney cell line HEK 293 responds to treatment with carnosine with a decrease in viability and ATP levels, although this reaction was less pronounced than in the glioblastoma cell lines. An increase of PDK4-mRNA expression after carnosine treatment has also been shown for this cell line [9]. HEK 293 is an immortalized embryonic cell line and therefore may not reflect the physiology of normal adult cells. It has even been argued that HEK 293 expresses proteins typically found in early differentiating neurons or neuronal stem cells, rather than normal epithelial kidney cells [132].

In conclusion, carnosine and its component L-histidine significantly decrease viability and ATP production in cell lines derived from human glioblastoma, with L-histidine exerting a markedly stronger influence. Also, both compounds alter PDK4-mRNA expression, with L-histidine again showing a more pronounced effect than carnosine. An influence on the expression of a human PDK4 promoter-reporter gene could not be detected. Even though it is tempting to speculate that carnosine may function as a reservoir for histidine, this assumption may be oversimplified.

## **5.2 Outlook and suggestions for further research**

In order to understand the mechanisms by which carnosine and L-histidine elicit their physiological effects in general and their influence on tumour cells in particular, an analysis of the mechanisms by which they interact with expression of PDK4-mRNA might be of interest. A further exploration of this issue may be an important step towards the identification of the molecular targets and signal transduction pathways affected by these two substances. Since the significant effect on PDK4-mRNA expression could not be mirrored in the reporter gene experiments, a direct interaction with the human PDK4 promoter region covered in the reporter gene is unlikely. Interactions of carnosine and histidine with epigenetic mechanisms such as histone acetylation or chromatin packaging on the other hand offer a possible explanation for these results, since epigenetic modifications of a circular reporter gene are likely to be different from the endogenous linear PDK4 gene. Further studies should therefore

investigate the influence of carnosine and histidine on such mechanisms. A comparison with the effects of agents known to influence histone acetylation and de-acetylation, such as the histone deacetylase inhibitor Trichostatin A, may shed further light on this issue. Assessing changes in histone acetylation after treatment with carnosine or histidine could also offer new perspectives on this matter. A direct interaction with histone structure could be possible as well as interactions mediated by other, so far unknown factors. In addition, it would be interesting to determine whether carnosine and L-histidine also affect PDK4 protein expression and PDK4 enzymatic activity as well as the activity of the pyruvate dehydrogenase complex.

With regard to a possible use of carnosine for therapy, the problem of its cleavage by CN1 in human serum, which reduces its bioavailability in the target tissues, has to be addressed. If carnosine should in fact just function as a vehicle for L-histidine, other histidine containing dipeptides which are not substrates of CN1 but of CN2 might offer a solution. Inhibition of CN1 might be an alternative. CN1 has been proposed as a medicinal target in neurological disorders by Vistoli *et al.* [133]. Despite this obstacle, an effect of orally administered carnosine on processes in the human brain has already been demonstrated [82].

In addition to this, the general observation that carnosine and its component L-histidine influence relevant metabolic enzymes such as PDK4 is interesting with regard to metabolic diseases, for example diabetes. Histidine might be a functional amino acid able to influence gene expression and thus alter the metabolic state of the organism, as has been shown for other amino acids such as arginine, leucine and proline [134].

The abundance of both carnosine and histidine in the human body and their complex metabolism can be seen as an indicator for their importance. Understanding the ways in which both substances interact with the metabolism of healthy and neoplastic cells remains as a promising objective for future research.



## 6 Summary

Dissertation zur Erlangung des akademischen Grades Dr. med.

Effects of carnosine and L-histidine on viability and expression of pyruvate dehydrogenase kinase 4 in human glioblastoma cells

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Since the dipeptide carnosine ( $\beta$ -alanyl-L-histidine) was discovered more than 100 years ago [30], a number of physiological functions have been attributed to it as well as a variety of beneficial pharmacological effects. However, the mechanisms underlying these effects are far from being understood. With regard to carnosine's anti-neoplastic effects first experiments using a Sarcoma-180 mouse model were presented by Nagai and Suda [1]. These experiments were later confirmed by mouse models with human colon cancer cells [83] and Her2/neu-positive NIH3T3-fibroblasts [2]. In addition, the anti-neoplastic effect was confirmed in tumour cell lines *in vitro*, in which also a selectivity towards neoplastic cells was demonstrated [4]. In the experiments performed by Renner *et al.* growth inhibition could also be shown for cells isolated from human glioblastoma [3]. This primary brain tumour is characterized by a highly malignant behaviour for which, despite its rather high incidence, therapeutic choices are limited. The inhibitory effect on the glioblastoma cells was linked to a reduction of ATP synthesis and the inhibition of glycolysis [7]. These findings turned the focus on enzymes of glycolysis as possible targets of carnosine. Experiments by Oppermann *et al.* showed a marked increase of mRNA encoding pyruvate dehydrogenase kinase 4 (PDK4), an enzyme involved in switching cellular metabolism under fasting conditions by phosphorylating the pyruvate dehydrogenase complex. The aim of the experiments presented in this thesis was to assess whether the effects of carnosine on cell viability and PDK4-mRNA expression could be attributed to its

component L-histidine and, in a further step, to identify possible cis-active responsive elements in the human PDK4 gene which mediate the effect of carnosine and L-histidine on expression.

Cell viability assays measuring ATP production, dehydrogenase activity and lactate dehydrogenase release into the medium were conducted on the three human glioblastoma cell lines U87, T98G and LN405 incubated with the substances of interest. The negative effect on viability and ATP levels elicited by carnosine could be reproduced. A similar, yet much stronger response could be shown after incubation with L-histidine. After incubation with carnosine's second component  $\beta$ -alanine, dehydrogenase levels were lowered, but only T98G responded with a decrease of ATP synthesis as well. Only L-histidine led to a rise in lactate dehydrogenase activity in the supernatant of the cultivated cells, which indicates necrotic cell death. It could thus be concluded that L-histidine mimics and exceeds the effect of carnosine on the viability of human glioblastoma cell lines, while  $\beta$ -alanine has less to no influence. A less intense negative effect of carnosine and L-histidine on viability of non-neoplastic human embryonal kidney cells was observed as well.

To address the question of whether L-histidine could be released from carnosine in the investigated cell lines, expression of mRNA encoding the two enzymes serum carnosinase (CN1) and tissue carnosinase (CN2) was measured in LN405, T98G and U87 cells by reverse transcription and qRT-PCR. These two enzymes are responsible for cleaving L-histidine from carnosine in serum or in the cytosol. The results of the glioblastoma cells were compared to results of parallel measurements in cDNA from normal brain tissue. It could be shown that all three glioblastoma cell lines express CN1 and CN2 mRNA. Thus, a general ability to cleave L-histidine from carnosine can be assumed for these cells, which in the meantime was confirmed by measurements of intracellular histidine after carnosine incubation [9]. Differences in expression of CN2 mRNA between the cell lines paralleled differences in susceptibility to carnosine treatment observed in the cell viability experiments. It is interesting to note that compared to normal brain tissue, the glioblastoma cells expressed markedly lower levels of both carnosinase enzymes, especially of CN1.

Since in earlier experiments by H. Oppermann, incubation with carnosine had been shown to enhance expression of endogenous PDK4-mRNA in glioblastoma cells [8], it was assessed whether the same could be found for L-histidine. To this end, cells of the U87 and T98G cell lines were subjected to mRNA isolation after 24 hours of incubation with L-histidine. The mRNA was reverse transcribed and analysed in qRT-PCR. This revealed an expression increase of PDK4-mRNA after L-histidine treatment, as reported before for carnosine. In further experiments with U87 cells, this increase could be elicited by concentrations as low as 7 mM L-histidine and was detectable 3 hours after starting incubation with 50 mM L-histidine.

In order to analyse whether the effects of L-histidine and carnosine on PDK4-mRNA expression are mediated by cis-regulatory elements within the PDK4 promoter region, a reporter gene with DNA

sequences from the PDK4 gene controlling the expression of the *Gaussia princeps* luciferase gene was constructed. This reporter gene contained a sequence from the human PDK4 gene reaching from -3968 bp upstream to +319 bp downstream from the transcription starting point and ending at the start codon (ATG). The start codon in this construct was now the start codon of the luciferase gene, whose expression can be quantified by luminescence measurement. Cells of the human glioblastoma cell lines U87 and T98G were transfected with this reporter gene or with a control plasmid, which did not contain the PDK4 sequence. Thus, responses to different concentrations of carnosine and L-histidine could be compared. To monitor cell viability, ATP production in the cells was measured as well. As a result it could be shown that neither L-histidine nor carnosine influenced the expression of the reporter gene. Instead, changes in expression were parallel to those found in cells transfected with the control plasmid and could be attributed to effects of histidine and carnosine on cell viability in general. The influence of carnosine and histidine on endogenous PDK4-mRNA expression can therefore be expected to be linked to factors which are not present in the reporter gene. Such factors include regulatory elements outside of the PDK4 promoter and 5' UTR sequence covered by the reporter gene or epigenetic mechanisms like histone acetylation. Further research on these factors, for example by immuno-precipitation or methylation assays, will be necessary to shed more light on this issue.

It can be concluded that L-histidine alone or as a component of carnosine can reduce viability of human glioblastoma cells and stimulate expression of endogenous PDK4-mRNA. Since the enhancement of expression could not be linked to regulatory elements in the PDK4 promoter and 5' UTR by reporter gene assay, an interaction of the two substances with other, namely epigenetic mechanisms appears likely. These findings reveal the diverse properties of carnosine and histidine and may contribute to our understanding of metabolism in neoplastic cells and to possible therapeutic uses of the still enigmatic dipeptide carnosine.

Parts of the results presented in this work have previously been published in *Amino Acids* as a joint work with H. Oppermann, J. Meixensberger and F. Gaunitz [9].

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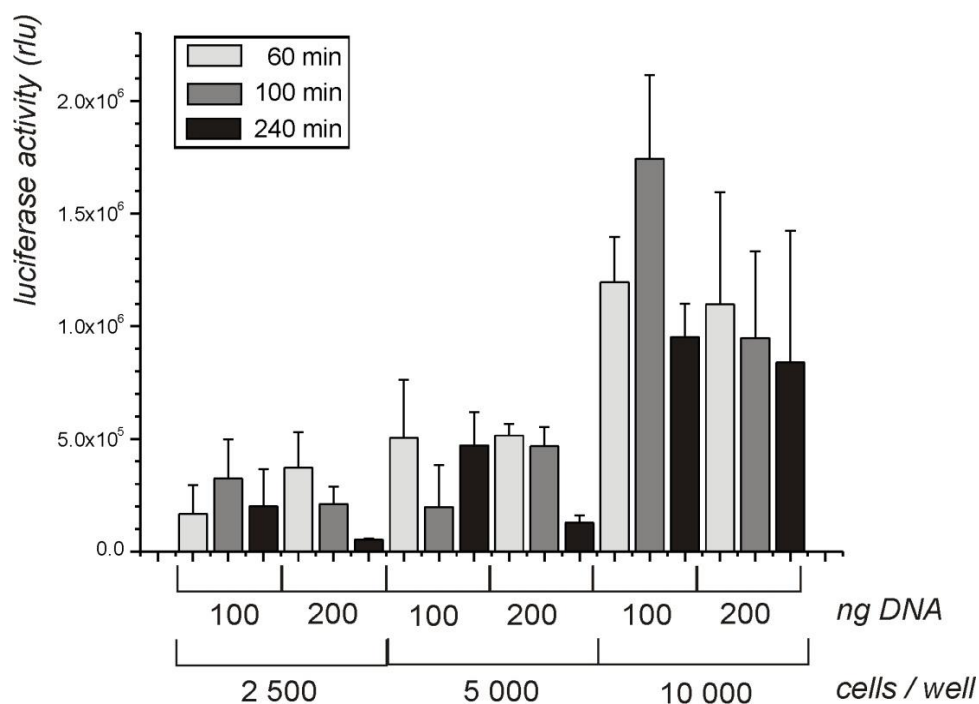


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## 8 Appendix - Optimization of transfection conditions for U87 cells

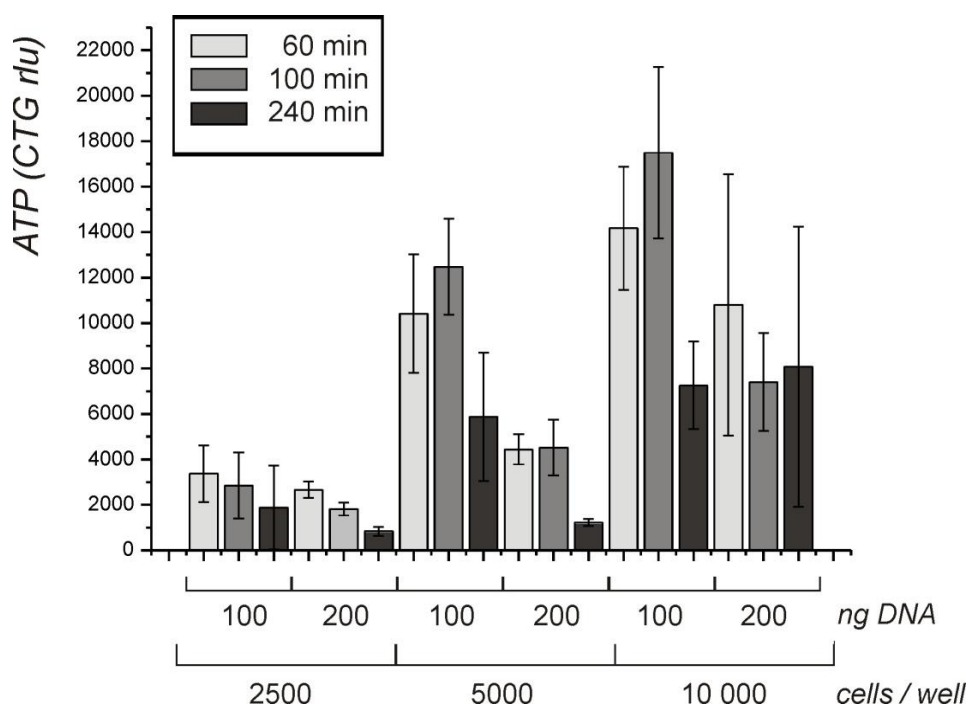
In order to optimize the transfection of cells of the line U87, cells at different densities were transfected with varying amounts of DNA and varying incubation times in the presence of DNA and the transfection reagent.

Cells grown close to confluence were detached from 75 cm<sup>2</sup> culture flasks using Accutase and seeded into the wells of a black 96-well Greiner microplate with clear bottom in 200 µl of DMEM at a density of 2,500, 5,000 and 10,000 cells per well. Twenty four hours later, the cells in each well received 200 µl of fresh medium with a previously prepared transfection mixture. This transfection mixture contained 100 or 200 ng of DNA (50% *pCMV\_hGLuc* and 50% *pNEGFP*) and the corresponding amount of 0.2 or 0.4 µl of the cationic polymer based transfection reagent Turbofect in 10 µl or 20 µl serum free medium. The transfection reagent was added by slow dripping. Then, the mixture was vortexed shortly and left to stand at room temperature for 20 minutes. Following this, it was expanded to a volume of 200 µl for each well by adding standard medium and used to replace the old medium on the cells. After an incubation time of 60, 100 or 240 minutes, the medium was exchanged with fresh standard medium. Twenty two hours later transfection efficiency and viability were estimated by fluorescence microscopy. From this first observation it became evident that cells plated at 2,500 and 5,000 cells per well should not be treated with DNA/transfection reagent for more than 100 minutes and with only 100 ng of DNA. Cells at a density of 10,000 cells per well appeared healthier under all conditions. For a quantitative determination of transfection efficiency 5 µl of supernatant from each well were transferred to a white microplate and *Gaussia princeps* luciferase activity was measured. The result of this experiment is presented in Figure 20. In order to evaluate whether the different conditions employed were harmful to the cells, ATP production by CellTiter-Glo™ assay was determined. The corresponding results are presented in Figure 21. As can be seen, cells cultivated at a density of 10,000 cells per well and incubated with 100 ng of DNA for 100 minutes appear to be most healthy regarding ATP production whilst at the same time showing the highest transfection efficiency. These parameters were adopted as a standard protocol for further transfection of U87 cells.



**Figure 20: *Gaussia* activity in U87 cells under varying transfection conditions**

Cells plated at a density of 2500, 5000 or 10000 cells per well on a 96-well plate were incubated for either 60, 100 or 240 minutes with a transfection mixture containing either 100 or 200 ng of DNA. Presented is *Gaussia princeps* luciferase activity as measured 22 hours after transfection by luminescence measurement. Error bars indicate standard deviation.



**Figure 21: Cell viability in U87 cells under varying transfection conditions**

Cells plated at a density of 2500, 5000 or 10000 cells per well on a 96-well plate were incubated for either 60, 100 or 240 minutes with a transfection mixture containing either 100 or 200 ng of DNA. Presented are ATP totals as measured 22 hours after transfection by CellTiter-Glo™ assay. Error bars indicate standard deviation.

## **Erklärung über die eigenständige Abfassung der Arbeit**

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

Leipzig, 19. 07.2015

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Datum

A handwritten signature in black ink, appearing to be 'U. Geh', written over a dotted line.

.....

Unterschrift

# Curriculum vitae

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**Primary education:** 1992 - 2004 Primary school and Gymnasium  
09 July 2004 Abitur

**University:** October 2004 - March 2005 Studying Biological Anthropology and Archeology at Friedrich-Schiller-University, Jena

October 2005 - September 2008 Studying Journalism and Arabic Studies at Leipzig University, before that working as a trainee journalist  
2006 head of news department of radio "mephisto 97.6"  
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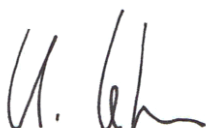
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Leipzig, 19 July 2015

## List of publications

### Papers:

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doi: 10.1007/s00726-014-1664-8

### Congress presentations:

Oppermann H, **Letzien U**, Meixensberger J, Gaunitz F (2014) The specific antineoplastic effect of carnosine and its dependence on release of L-histidine under the influence of carnosinase in glioma tumor cells. 65. Jahrestagung der Deutschen Gesellschaft für Neurochirurgie.  
doi: 10.3205/14dgnc053

### Posters:

**Letzien U**, Oppermann H, Meixensberger J, Gaunitz F (2014) Transcriptional effects of carnosine in glioblastoma cells are mimicked by the histone deacetylase inhibitor Trichostatin A. 65. Jahrestagung der Deutschen Gesellschaft für Neurochirurgie. doi: 10.3205/14dgnc465

Oppermann H, **Letzien U**, Meixensberger J, Gaunitz F (2013) The specific antineoplastic effect of carnosine and its dependence on release of L-histidine under the influence of carnosinase in tumor cells. 12. Research Festival for Life Sciences Leipzig

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